Innate Immune Mechanisms in the Pathogenesis and Management of Acute Pancreatitis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by Peter Szatmary

Gott hat niemals eine Krankheit entstehen lassen, für die Er nicht auch eine Arznei geschaffen hätte.

Paracelsus

God has never permitted a disease to exist for which he has not also created a treatment.

Abstract

Acute pancreatitis is a common, debilitating condition characterised in its most severe form by organ necrosis as well as systemic inflammation, organ failure and death. The innate immune system, predominantly mediated through neutrophils, has been shown to contribute significantly to both pancreatic injury and respiratory failure: the leading cause of death in severe acute pancreatitis. Neutrophil extracellular traps, anti-microbial nuclear chromatin laced with proteases, are a recent discovery and contribute to the genesis of extracellular free radicals, microvascular thrombosis and abscess formation.

Histones and histone fragments are critical active components of neutrophil extracellular traps. They are amongst the most widely conserved biochemical structures, are released as antimicrobial agents by a wide range of organisms and are uniquely toxic to cellular organisms. A central hypothesis for this work, is that the release of neutrophil extracellular traps, and their histone component in particular, is central to the way by which the innate immune response exacerbates acute pancreatitis.

By using a 12-injection model of cerulein pancreatitis in mice depleted of specific innate immune cell subsets (Ly6G neutrophils, Ly6C monocytes and CCR2hi inflammatory monocytes), cytokine feedback mechanisms were identified and differential effects on pancreas and lung demonstrated by different cell types. In particular, CCR2hi pro-inflammatory monocytes appeared to contribute greatest to pancreatic injury, whereas Ly6Ghi neutrophils contributed most to lung injury.

Using selective simultaneous measurement of portal, systemic venous and arterial blood, the liver was identified as the predominant source of circulating histones in acute pancreatitis, although hepatic immune cells are likely responsible for this signal amplification, similar to that seen with cytokines. Systemic injection of FITC-labelled histones demonstrated concentration within the pancreas only in concomitant pancreatitis. In (acinar) cellular isolates, histones led to dose-dependent necrosis by disrupting the plasmalemma. This in turn led to calcium currents across the membrane, which were not necessary to achieve cytotoxicity.

By developing novel methodologies of measuring perfusion on a whole-organ level in mouse pancreas, an overall reduction in pancreatic perfusion is observed in cerulein AP, with patches of ischaemia resulting in focal necrosis on the same time-scale as maximal histone release. Pre-treatment with NETosis inhibitors reduced pancreatic injury, but histone detoxification as treatment (3-hours after induction of acute pancreatitis) was not effective.

These results indicate modulation of innate immune mechanisms may allow future therapies to be targeted selectively at different phases of acute pancreatitis, to control extra-pancreatic effects and organ failure independently from pancreatic injury. Significant challenges remain to deliver therapies to a poorly perfused pancreas.

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Declaration of Academic Honesty

I hereby confirm that:

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Contents

Abbreviations

A

ALT - Alanine Aminotransferase 48, 81, 88

AP - Acute Pancreatitis 26, 29, 42, 88, 89, 123, 126

ATP - Adenosine Triphosphate 31

B

BSA - Bovine Serum Albumin 28, 50

C

CBA – Cytokine Bead Assay 60

CCL2 - C-C Ligand 2 ..... 60, 72

CCR2 - C-C Chemokine Receptor 2 ..... 72, 74

CFTR - Cystic Fibrosis Transmembrane Regulator 28

CG - Cathepsin G 35

CRP - C Reactive Protein 76, 79, 88, 94

CXCL-1 - C-X-C Ligand 1 ..... 60, 72

CXCR2 - C-C Chemokine Receptor 2 35

D

DAMP - Damage Associated Molecular Pattern 28, 31, 33, 53, 73, 89, 126, 146, 159

DAMPs - damage associated molecular patterns 154

DIC - Disseminated Intravascular Coagulopathy 40

DNA - Deoxyribonucleic Acid 36, 37, 38, 39, 40, 93

E

EDTA - Ethylenediaminetetraacetic Acid 49, 55, 56

ELISA - Enzyme Linked Immunosorbent Assay 77, 78

ERCP - Endoscopic Retrograde Cholangiopancreatography 27

F

FASP - Factor VII-Activating Protease 138

FITC - Fluorescein Isothianate 48, 78, 86, 87, 90, 100

Fluo4-AM – 4-(6-Acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4'-methyl-2,2'-(ethelynedioxy)dianiline-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl) ester 102

Fura-2-AM - Fura 2 Pentakis(Acetoxymethyl) Ester 102

H

HDAP - Histone Derived Antimicrobial Peptide 38

HMGB1 - High Mobility Group Box Protein 1 ..... 31, 146, 157

I

ICAM-1 - Intercellular Adhesion Molecule 1 53

IFNγ - Interferon gamma 60

IL-1β - Interleukin 1 beta 60, 72, 157

IL-6 – Interleukin 6 60

IL-8 - Interleukin 8 33

IQR - Interquartile Range 51

K

KC - Keratinocyte Chemoattractant 72

L

LMWH - Low Molecular Weight Heparin 131, 134

LPS - Lipopolysaccharide 33, 35, 154, 160

M

Mac-1 - Macrophage-1 Antigen 34

MCP-1 - Monocyte Chemoattractant Protein 1 72

MPO - Myeloperoxidase 34, 36, 46, 53, 70, 84, 87

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1 Introduction

1.1 Acute pancreatitis

1.1.1 Disease burden

Acute Pancreatitis (AP) is an acute inflammatory disease of the exocrine pancreas, for which a disease-modifying, specific therapy is yet to be found. Despite several hundred randomised controlled trials the mainstay of therapy remains supportive, with modest improvements in mortality resulting from improvements in care pathways and critical care medicine (Godi, Eross et al. 2018; Mandalia, Wamsteker et al. 2018).

AP is diagnosed through a combination of clinical evaluation, imaging and biochemical analysis (IAP/APA Working Group 2013). It has an incidence ranging from 4.6 to 100 cases per 100,000 population per year in Europe (Roberts, Morrison-Rees et al. 2017) and occurs at similar frequencies in other Westernised nations (Yadav and Lowenfels 2013; Pendharkar, Mathew et al. 2017). It is one of the commonest specific gastrointestinal disorder leading to hospitalisation and annual costs escalating from $2.5 billion to $9.2 billion (1997 versus 2012) in the US alone (Peery, Dellon et al. 2012; Wadhwa, Patwardhan et al. 2017). Although the initial injury is confined to the pancreas, a subset of patients develop systemic disease contributing to significant morbidity and mortality (Banks, Bollen et al. 2013).

This work sets out to better understand the inflammatory processes that form the link between pancreatic injury and organ failure, ultimately the cause of mortality in patients with severe AP. It further aims to describe mechanisms

that can be targeted on a clinically-relevant time-scale and form the basis of the development of therapy that can alter the course of the disease.

1.1.2 Pathophysiology

The first reported death from necrotizing acute pancreatitis was in 1760, by the Italian anatomist Giovanni Battista Morgagni and the first description of an experimental model of acute pancreatitis by Claude Bernard through retrograde infusion of bile into the pancreatic duct followed in the early 19th century (Keminger 2001). The first documented case of human AP of known aetiology by Eugene Opie in 1901 was as a result of a gallstone (Lerch, Weidenbach et al. 1994). While research into the pathophysiology of acute pancreatitis continued thereafter, only the advances in cell biology, immunology and imaging of the late 20th century allowed detailed understanding of the complex interactions that result in pancreatic inflammation, necrosis and organ dysfunction.

Gallstones and alcohol are now the commonest aetiological factor for acute pancreatitis in Europe (Parniczky, Kui et al. 2016; Roberts, Morrison-Rees et al. 2017), with hyperlipidaemia, idiopathic and post-ERCP pancreatitis accounting for nearly all remaining cases. Associated factors including obesity (Ikeura, Kato et al. 2017; Yoon, Choi et al. 2017) and smoking (Kim, Chung et al. 2017; Stigliano, Belisario et al. 2017) exacerbate the severity of each episode of acute pancreatitis and increase the likelihood of recurrent episodes. On a cellular level, injury to pancreatic acinar cells leads to influx and overload of intra-cellular calcium (Voronina, Collier et al. 2015; Wen, Voronina et al. 2015), which in turn promotes mitochondrial dysfunction and cell death (Mukherjee, Mareninova et al. 2016). Activation of zymogens

occurs within the pancreatic parenchyma and propagates (or initiates, in the case of hereditary pancreatitis) cellular injury (Van Acker, Weiss et al. 2007; Athwal, Huang et al. 2014). Activated lipase causes lipolysis of visceral fat, providing a putative mechanism of how obesity exacerbates disease severity (Patel, Trivedi et al. 2015). Alcohol and cigarette smoke further contribute to disease severity by inhibiting CFTR (Hegyi, Wilschanski et al. 2016), thereby limiting chlorine currents, ductal secretion and clearance of activated proteases (Kadiyala, Lee et al. 2013). Injured acinar cells release a cascade of chemokines and cytokines, while necrotic cells release DAMPs which lead to the recruitment of immune cells and initiation of a local and systemic inflammatory response (Gukovskaya, Gukovsky et al. 2017).

1.1.3 Challenges investigating human AP

Due to the anatomical constraints of a gland situated deep within the peritoneal cavity, surrounded by major vasculature (Tsuchitani, Sato et al. 2016) and the likelihood that many disease-relevant changes occur prior to presentation to hospital (Kambhampati, Park et al. 2014; Afghani, Pandol et al. 2015), use of experimental models is critical in AP. Significant differences in digestive systems of vertebrates, however, mean mammalian models are indispensable, allowing meaningful comparison to human disease. Even within mammals, however, there are significant anatomical differences between the primate pancreas and the more commonly utilised experimental animals – rodents (Tsuchitani, Sato et al. 2016). Because of their low maintenance costs and availability of powerful genetic tools, rodents have become the mainstay for pancreatitis research. Nevertheless, there are further differences between baseline metabolic and immune functions

between rodents and human that lead to documented differences in response to other systemic inflammatory diseases such as sepsis (Iskander, Osuchowski et al. 2013; Fink 2014) or trauma (Frink, Andruszkow et al. 2011).

To investigate organ-specific effects such as organ necrosis or inflammatory infiltration, or systemic responses such as organ failure, *in vivo* models of AP are used. The commonest of these aim to replicate the common aetiologies of AP in humans, biliary obstruction by gallstones and chronic alcohol excess (Frossard, Steer et al. 2008) and include invasive techniques to obstruct bile duct drainage or inject bile acids into the pancreatic duct (Perides, van Acker et al. 2010; Wan, Huang et al. 2012) or injection of ethanol and fatty acid metabolites (Criddle, Raraty et al. 2004). While these models replicate human disease in principle, they have the disadvantage of necessitating anaesthesia and surgery (bile acid model) or injection of high concentrations of ethanol or solvent which in turn lead to local and systemic effects of the injection vehicle. The commonest *in vivo* model of experimental acute pancreatitis is hyperstimulation by infusion or repeated injections of the cholecystokinin analogue cerulein (Saluja, Saito et al. 1985). While this model is not directly representative of human disease, it has the advantage of producing dose-sensitive pancreatic inflammation without mortality, is cheap and reproducible.

Investigating pathophysiological events on a cellular level can often be done in *in vitro* and *ex vivo* preparations of isolated pancreatic acinar cells (Williams, Korc et al. 1978; Orabi, Muili et al. 2013), acinar cell lobules (Flowe, Welling et al. 1994) or slices (Huang, Gaisano et al. 2011) and isolated, perfused pancreas (Otsuki, Sakamoto et al. 1979; Reaven, Curry et al. 1983).

Pancreatic acinar cells rapidly lose their exocrine phenotype and trans-differentiate into ductal-type cells in culture (Hall and Lemoine 1992; De Waele, Wauters et al. 2014), necessitating fresh harvest of cells for each experiment. However, acinar cells from any species (including human) can be interrogated in this manner.

1.1.4 Inflammation links pancreatic necrosis to organ failure

At the first international consensus meeting on acute pancreatitis in Atlanta in 1992, pancreatic necrosis was deemed sufficient to categorize acute pancreatitis as severe (Bradley 1993). However, a realisation that necrosis in the absence of organ failure probably did not by itself contribute to a significant increase in mortality (Ashley, Perez et al. 2001) led to a revision of the original Atlanta Classification (Banks, Bollen et al. 2013) which downgraded the significance of pancreatic necrosis in the absence of organ failure and led to the development of a rival classification system, which eliminated the inclusion of pancreatic necrosis as a determinant of severity entirely (Dellinger, Forsmark et al. 2012).

Organ failure is rarely a global phenomenon in acute pancreatitis. The lung is generally the first or only organ to fail (Szatmary, IAP Presentation 2015) and is an independent predictor or mortality (Skouras, Hayes et al. 2014; Parniczky, Kui et al. 2016). Lung injury in AP can be direct, through circulating toxins and activated pancreatic proteases bypassing the liver through the thoracic duct in mesenteric lymph (Mittal, Phillips et al. 2009), or indirect through the injurious effects of innate immune cells.

Pancreatic acinar cell (PAC) injury induces synthesis and release of pro-inflammatory cytokines (Gukovskaya, Gukovsky et al. 1997; Szatmary and Gukovsky 2016) and PAC death releases pro-inflammatory DAMPs (including histones, HMGB1 and ATP) (Kang, Lotze et al. 2014). Extracellular nuclear proteins can be measured as early as four hours after induction of experimental acute pancreatitis (Ou, Cheng et al. 2015; Schneider, Jabrailova et al. 2015) and act via common immune sensors and initiators including toll-like receptors (TLRs, nucleotide binding domain (NOD)-like receptors (NLRs) and receptor for advanced glycation end-products (RAGE) to initiate sterile inflammation (Chen and Nunez 2010).

This initial response generates an early cellular response consisting of glandular infiltration with neutrophils and monocytes, which appear to exacerbate injury (Frossard, Saluja et al. 1999; Gukovskaya, Vaquero et al. 2002), act together with peritoneal macrophages and hepatic Kupffer cells to amplify pro-inflammatory cytokines in experimental acute pancreatitis (Gloor, Todd et al. 1998; Gloor, Blinman et al. 2000; Lundberg, Eubanks et al. 2000), and are at least in part responsive for early onset organ failure seen in some acute pancreatitis patients (Oiva, Mustonen et al. 2010; Oiva, Mustonen et al. 2013).

1.1.5 Therapeutic Strategies

Very few therapeutic trials in human acute pancreatitis have shown promise and even fewer ultimately successful, underlining the difficulties faced by researchers hoping to translate animal studies into human treatments. A prime example of this are the lexipafant trials – a series of randomised controlled trials of a platelet activating factor inhibitor (Kingsnorth, Galloway et al. 1995; McKay, Curran et al. 1997; Wyncoll and Beale 1998; Johnson, Kingsnorth et al.

2001), which were powered on crude endpoints such as mortality and reduction in proportion of complications and included patients up to 72 hours after onset of the disease and often with established organ failure. Nevertheless, they demonstrated a tendency to a reduction in severity of organ failure.

Inhibitors of pancreatic proteases have been developed and are used in clinical practice, predominantly in Japan, given intra-venously or as continuous regional arterial infusion alone and in conjunction with antibiotics with mixed effects. Despite promise in some observational studies, larger cohort studies and meta-analyses have shown no benefit (Hamada, Yasunaga et al. 2013; Horibe, Egi et al. 2015; Horibe, Sasaki et al. 2017), and use is not recommended in the latest version of the Japanese guidelines for the management of acute pancreatitis (Yokoe, Takada et al. 2015). Experimentally, gabexate mesilate and nafamostat mesilate (two sereine protease inhibitors in clinical use) infusions improved pancreatic blood flow and increased capillary density in rats with severe acute pancreatitis (Keck, Friebe et al. 2005). This effect, however, has only been demonstrated in concomitant- or pre-treatment.

A recently discovered innate immune mechanism - neutrophil extracellular traps (NETs) - potentially links these trials. Their release is stimulated through the interaction with activated platelets and regulated by neutrophil elastase: another serine protease. The next sections describe the biology of NETs and the role of histones, one of their principle structural components.

1.2 Neutrophil extracellular traps

1.2.1 Composition

Neutrophil extracellular traps, or NETs, were first described in 2004 as fragile fibres released by neutrophils in response to IL-8, LPS or PMA (Brinkmann, Reichard et al. 2004). NETs are composed of DNA strands, linked by histones and laced with neutrophil elastase, cathepsin G and myeloperoxidase. The release process is dependent on NADPHO and results in nuclear chromatin decondensation, translocation of granular proteins to the nuclear material, disintegration of the nuclear envelope and release of formed NETs (Fuchs, Abed et al. 2007).

1.2.2 Release Mechanisms

A large number of signals have been shown to be able to induce NET, including bacteria (Berends, Horswill et al. 2010; Juneau, Pang et al. 2011; Young, Malcolm et al. 2011), viruses (Narasaraju, Yang et al. 2011; Saitoh, Komano et al. 2012), yeasts (Urban, Reichard et al. 2006; Bruns, Kniemeyer et al. 2010), parasites (Baker, Imade et al. 2008; Abi Abdallah, Lin et al. 2012), organic crystals (Mitroulis, Kambas et al. 2011; Schauer, Janko et al. 2014), non-organic matter (Bartneck, Keul et al. 2010; Jhunjhunwala, Aresta-DaSilva et al. 2015), cytokines (Keshari, Jyoti et al. 2012; Rossaint, Herter et al. 2014) and cellular breakdown products including DAMPs (Tadie, Bae et al. 2013; Huang, Tohme et al. 2015). To detect such a varied set of stimuli, there is overlap and convergence of receptor pathways. This may explain some variability in early genetic knock-out studies when defining which receptor is critical in mediation NET release. It would seem molecular pattern-related NET release is

mediated predominantly through toll-like receptors (TLR) 2, 4 and 9 (Clark, Ma et al. 2007; Allam, Scherbaum et al. 2012; Semeraro, Ammollo et al. 2014; Huang, Tohme et al. 2015), immune complex-related NET release is mediated via Fc receptors and MAC-1 (Behnen, Leschczyk et al. 2014) and larger pathogens or inorganic matter lead to NETosis through sheer size. The inability to phagocytose large particles within a given time appears to drive neutrophils to auto-digest and release NETs in a process dependent on dectin-1 (Branzk, Lubojemska et al. 2014).

Following signal detection, there are three critical steps leading to NET release: phagocyte oxidase (PHOX/NADPHO) activation, nuclear protease translocation and histone de-imination.

Involvement of PHOX/NADPHO is illustrated by patients with chronic granulomatous disease, an inherited defect in PHOX activity, who are unable to produce NETs when stimulated with phorbol 12-myristate 13-acetate (PMA) and present with recurrent and/or persistent infections(36). PHOX/HADPHO is activated by protein kinase C (PKC). Experimentally, pan-activation of PKC isoforms using PMA or the di-acyl glycerol analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG) effectively stimulates NETosis (Gray, Lucas et al. 2013). Inhibition of NADPHO or myeloperoxidase (MPO) effectively inhibits NETosis stimulated by PMA, whereas inhibition of mitochondrial respiration or superoxide dysmutase does not (Kirchner, Moller et al. 2012). Specific inhibition of PKC isoform β inhibits both reactive oxygen species (ROS) production by PHOX and NETosis, however there are conflicting reports on whether PKCζ is also able to inhibit NETosis (Gray, Lucas et al. 2013; Neeli and Radic 2013). There are increasing reports of NADPHO-independent NETosis, such as via the

src kinase family in response to chemokine receptors (CXCR2) activation (Marcos, Zhou et al. 2010) or via unspecified pathways following high-dose uric-acid stimulation (Arai, Nishinaka et al. 2014).

Histone de-imination by peptidyl-arginine transferase 4 (PAD4) is an essential step towards NET release (Neeli and Radic 2012). PAD4 targets methyl-arginine residues, reducing methylation and increasing citrullination on H4Arg3 and H3Arg2, 8 and 17 residues in HL-60 cells (Wang, Li et al. 2009) over a time scale of 15 minutes to 2 hours, in a manner independent of caspase activity (Neeli, Khan et al. 2008). These same post-translational modifications are amongst the most immunogenic histone modifications seen in serum from patients with systemic lupus erythematosus (Liu, Tangsombatvisit et al. 2012), and levels of circulating nucleosomes and citrullinated histone H3 correlate with disease severity in acute inflammatory conditions including sepsis (Hirose, Hamaguchi et al. 2014), trauma (Abrams, Zhang et al. 2013) and pancreatitis (Ou, Cheng et al. 2015). Genetic deletion of PAD4 leads to an inability of neutrophils to release NETs in response to calcium ionophore treatment or LPS (Martinod, Demers et al. 2013) and pharmacological inhibition of PAD4 inhibits NET formation in murine and human neutrophils (Lewis, Liddle et al. 2015). Overexpression of PAD4, on the other hand, has been shown to cause histone hypercitrullination, nuclear de-condensation and release of NET like structures in an osteosarcoma cell line (Leshner, Wang et al. 2012).

Nuclear translocation of granular proteases is the final step towards NET release. Neutrophil azurophilic granules contain neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G (CG), however only NE is translocated to the nucleus and neither inhibition of PR3 nor CG can prevent this translocation

(Papayannopoulos, Metzler et al. 2010). Furthermore, the process is not mediated by fusion of granules with the nucleus, but rather NE dissociates from the granular membrane in a ROS-dependent manner before degrading cytosolic actin, arresting actin dynamics and translocating across the nuclear membrane using specific translocation mechanisms (Metzler, Goosmann et al. 2014). Binding of nucleic acid by proteases initiates a process of degradation of nuclear binding proteins(Thomas, Whangbo et al. 2014) and controlled integration of MPO into the forming NET. Nuclear NE leads to early degradation of linker histone H1, followed by core histone H4 which coincides with nuclear chromatin de-condensation (Papayannopoulos, Metzler et al. 2010). Histone H3 appears to be resistant to degradation in intact nuclei, but not in purified form, suggesting one of the purposes of post-translational modification is to render histone H3 resistant to NE related degradation.

1.3 Histones

1.3.1 Biochemistry

Histones are positively charged nuclear proteins which help to package DNA into nucleosomes common to all eukaryotic cells. They were first described by Albrecht Kossel as histidine-rich peptones (his-tones) derived from avian red blood cell nuclei in 1884 (Kossel 1884); he was awarded the Nobel Prize in Physiology and Medicine for this and other work on the nucleus of cells in 1910. The structures and sequences of histones are highly conserved across all eukaryotic species (McGhee and Felsenfeld 1980; Talbert and Henikoff 2010), and allow them to act as nuclear chaperone proteins, interacting with nucleic acids due to their highly net-positive charge (Park and Luger 2008) from

arginine and lysine residues (Sugano, Olson et al. 1972; Yeoman, Olson et al. 1972). Each nucleosome particle consists of 147 base pairs of DNA, wrapped in 1.7 turns around a protein octamer of core histones (H2A, H2B, H3 and H4), further compacted by linker histones H1 and/or H5 (Luger, Mader et al. 1997).

Numerous post-translational modifications of histones have been identified to date, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination and proline isomerisation (Kouzarides 2007). In normal cell function, these alter the nature of the histone-DNA interaction and allow transcription to occur.

More recently, controlled histone degradation has been described in neutrophils leading to chromatin de-condensation and release of genomic DNA laced with granular proteins as neutrophil extracellular traps (NETs) (Brinkmann 2004; Papayannopoulos, Metzler et al. 2010). These meshwork-like structures promote intravascular thrombosis (Fuchs, Brill et al. 2010), limit spread of micro-organisms, encourage cancer metastasis (Cools-Lartigue, Spicer et al. 2013) and cause direct injury to adjacent cells (Allam, Kumar et al. 2014).

1.3.2 Extracellular Histones in Disease

Two types of disease process are associated with a significant increase in circulating histones: systemic infections and ischaemic processes. In sepsis in particular, extracellular histones have been identified as viable biomarkers and anti-histone strategies are being trialled as potential therapies (Xu, Huang et al. 2015). The seemingly lacking connection between infective and ischaemic/thrombotic diseases can be reconciled by the introduction of the

concept of immune-thrombosis. Histones, released as part of NETs, induce microvascular thrombosis to limit the spread of micro-organisms (McDonald, Urrutia et al. 2012), but the undifferentiated response in sterile inflammation leads to exacerbation of the initial injury. This phenomenon can be seen in a range of human diseases, including stroke (Geiger, Holdenrieder et al. 2007; De Meyer, Suidan et al. 2012), heart attack (Savchenko, Borissoff et al. 2014), acute lung injury (Saffarzadeh, Juenemann et al. 2012) and ischaemic renal injury (Rosin and Okusa 2012).

1.3.3 Histone-mediated Cell Injury

A wide variety of organisms release histones and their degradation products as microbicidal agents in the form of histone-derived antimicrobial peptides (HDAP), with their activity documented *in vitro (Tagai, Morita et al. 2011; Wang, Chen et al. 2011)* as well as in animal (Drab, Kracmerova et al. 2014) and human (Kim, Cho et al. 2002) disease.

Exogenous histones appear to affect cells (including microbes) in one of two ways: permeabilization of membranes, or DNA-binding and disruption of transcription. In human disease, cellular injury by histones has been described in lung (Bosmann, Grailer et al. 2013), heart (Kalbitz, Grailer et al. 2015; Alhamdi, Zi et al. 2016), liver (Xu, Zhang et al. 2009; Huang, Chen et al. 2013), kidney (Allam, Scherbaum et al. 2012) and vascular endothelium (Saffarzadeh, Juenemann et al. 2012). Histone concentrations below 10 μg/ml seem to have a signalling function and can induce calcium transients in cells (Gamberucci, Fulceri et al. 1998). Concentrations greater than 10 μg/ml (or 20 μg/ml in the presence of serum) induce cell death by an uncertain mechanism, probably involving the formation of non-selective cationic pores

in membranes (Kleine, Lewis et al. 1997; Lete, Sot et al. 2014). Bactericidal properties of histone fragments are dependent on the ability to form amphipathic alpha-helices – potentially membrane spanning domains – however no such structural analyses have been performed on mammalian cells to date (Koo, Kim et al. 2008).

The interaction of histones with cell membranes is heavily reliant on charge. Positively charged histones preferentially bind anionic phospholipids such as cardiolipin or phosphatidylserine, but not zwitterionic phospholipids like phosphatidylcholine (Pereira, Marco et al. 1994). Furthermore, adding negative change (e.g.: a phosphate head group as in phosphatidylinositol-bis-phosphate) increases the binding capacity of histones as measured by calorimetry (Lete, Sot et al. 2014). Histones have also been shown to expose phosphatidyl serine on the surface of red blood cells in a dose-dependent manner (Semeraro, Ammollo et al. 2014), however it is unclear whether this resulted from altered flippase kinetics or via induction of apoptosis pathways.

Negatively charged acute-phase proteins (such as C-reactive protein) (Abrams, Zhang et al. 2013), DNA (Lete, Sot et al. 2014), polysaccharides (Fuchs, Bhandari et al. 2011) or synthetic macromolecules (Gamberucci, Fulceri et al. 1998) compete with membrane phospholipid-binding and prevent histone integration and toxicity.

1.3.4 Immunothrombosis

Recent data suggests that histones, both in isolation and as components of NETs, can influence the coagulation cascade to initiate venous thrombosis (Fuchs, Brill et al. 2010; Brill, Fuchs et al. 2012; von Bruhl, Stark et al. 2012).

Furthermore, circulating nucleosomes are associated with and independent prognostic markers of disseminated intravascular coagulopathy (DIC) (Kim, Lee et al. 2015), leading to some countries, notably Japan, to actively promote the use of anticoagulants as histone detoxification agents in the treatment of DIC (Iba, Gando et al. 2014). Positive correlation between histone levels and coagulopathy can also be seen in trauma patients (Kutcher, Xu et al. 2012) and patients with sepsis (Wildhagen, Wiewel et al. 2015).

Histones act synergistically to produce a profound pro-coagulant state. Histones are able to induce platelet aggregation, factor V/Va expression and prothrombinase activity, leading to thrombin activation independent of the intrinsic coagulation pathway (Semeraro, Ammollo et al. 2011). Histones also inhibit thrombomodulin and protein C activation (Ammollo, Semeraro et al. 2011), an effect most pronounced with histones H3 and H4, thus reducing a natural inhibitor system. Furthermore, histone H4 binding promotes thrombin auto-activation, probably by fixing the prothrombin molecule in a conformational state conducive to proteolytic attack (Barranco-Medina, Pozzi et al. 2013). The only exception is liker histone H1, which has been shown to reduce thrombin activation and prolong clotting times (Kheiri, Fasy et al. 1996); this mechanism is likely insignificant in acute inflammation, as histone H1 is amongst the first nuclear proteins to be degraded in the process of NETosis.

The presence of DNA in NETs also allows activation of the intrinsic coagulation pathway, demonstrated by NET-enhanced thrombin generation in platelet-poor plasma, reduced by factor XII/factor XI depletion or DNAse

treatment(Gould, Vu et al. 2014). DNAse treatment in platelet-rich plasma further increases thrombin generation, indicating differential effects of histones and NETs in different microenvironments. The addition of histones and DNA also increases fibrin fibre thickness, clot stability and delayed clot lysis (Longstaff, Varju et al. 2013) as well as reducing anti-thrombin-mediated thrombin inactivation and plasmin activity (Varju, Longstaff et al. 2015). In *in vivo* systems, the interplay between von Willebrand factor (vWF), platelets and neutrophils anchor neutrophils to otherwise healthy vessel walls and permit NETing neutrophils to initiate clot formation (Kolaczkowska, Jenne et al. 2015; Slaba, Wang et al. 2015), with propagation presumably aided by the mechanisms described above. Deficiencies in degradation of vWF produce clinical microangiopathies (e.g. thrombotic thrombocytopaenic purpura), the severity of which is also closely correlated with circulating NET components in humans (Fuchs, Kremer Hovinga et al. 2012).

1.4 Aims and hypotheses

To investigate the role of innate immunity in acute pancreatitis and the resulting systemic inflammatory syndrome, the following hypotheses and aims were generated.

1. Pancreatic injury results in recruitment of innate immune cells, resulting in an exacerbation of injury. Therefore:
    a. depletion of specific pro-inflammatory cell types (monocytes and/or neutrophils) will ameliorate pancreatic injury.
    b. there is a negative feedback loop by which inflammation is contained and total destruction of the exocrine pancreas avoided.

Using antibody-mediated depletion of specific myeloid subsets in experimental acute pancreatitis and measuring panels of inflammatory cytokines, these hypotheses have been addressed in work described in chapter 3.

2. Histones and histone fragments interact directly with pancreatic acinar cells to increase membrane permeability and cell death. Histones are generated within the pancreas through necrosis and/or active release by infiltrating immune cells, exacerbating injury to surrounding tissues. From this follows that:
    a. there is a measurable increase in calcium permeability of acinar cells in response to histone treatment.
    b. histone treatment of freshly isolated pancreas results in cellular necrosis that is in proportion to exposure.
    c. histone concentrations are highest in the portal circulation.
    d. histone concentrations correlate with disease severity.

    The aim here has been to address the above hypotheses in a manner relevant to clinical pancreatitis, yet consistent with the principles of the three Rs. Therefore, human serum levels have been measured to correlate histone levels with disease severity in chapter 4. Portal histone levels have been measured in an experimental model, also described in chapter 4, and effects on membrane integrity evaluated in cellular isolates in chapter 5.

3. Acute pancreatitis features small and medium-vessel thromboses, resulting in impaired microvascular perfusion in severe AP. Preventing NETosis or

detoxification of histones with heparin ameliorates AP on a cellular and whole-organ level.

Small-medium vessel perfusion studies in mouse are difficult to achieve. Nevertheless, this study aims to develop novel assays to evaluate the perfusion of small and medium sized blood vessels on a whole-organ level, allowing visualisation of patchy perfusion, as described in chapter 6. These methods have then been used to evaluate potential therapies in the treatment of acute pancreatitis as described in chapter 7.

2 Common Materials and Methods

2.1 Human tissue experiments

Blood Samples

Patients with acute pancreatitis included in the National Institute of Health Research Liverpool Pancreas Biomedical Research Unit Acute Pancreatitis Biobank were selected at random and plasma samples obtained as approved by the regional ethics committee (REC 10/H1308/31). All adult (18-99 years of age) patients attending the Royal Liverpool University Hospital with a diagnosis of acute pancreatitis of any aetiology (amylase >450 IU, typical pain and/or pancreatic inflammation on cross-sectional imaging) were eligible for inclusion to the biobank. Patients who were unable to consent (e.g. unconscious), had a history of recurrent acute or chronic pancreatitis or a history of pancreatic surgery or malignancy were excluded. Samples were collected prospectively within 24 hours of admission from consenting patients who had presented within 72 hours of onset of pain, together with clinical data that allowed severity stratification according to the 2012 Revised Atlanta Classification (Banks, Bollen et al. 2013). All samples were processed within 30 minutes of blood sampling and stored at 80 °C. Collection, processing, storage, monitoring and usage of samples followed pre-defined standard operating procedures and Good Clinical Practice.

2.2 Experimental acute pancreatitis

Experimental Animals

All animal studies were ethically reviewed and conducted as per the UK Animals (Scientific Procedures) Act 1986 under a project license approved by the UK Home Office (PPL 70/8109). Male C57BL/6J mice (age 8-10 weeks; 20-25 g) and Male CD1 Swiss mice (age 10-12 weeks; 30-35 g) were purchased from Charles River UK Ltd (Margate, Kent, UK), housed in a pathogen-free unit with 12 h light-dark cycles and had free access to standard lab chow and water.

In vivo models

Mild oedematous acute pancreatitis was induced by 4 hourly (Ou, Cheng et al. 2015) and necrotising acute pancreatitis by 12 hourly (Huang, Cane et al. 2015; Ou, Cheng et al. 2015) intraperitoneal injections of cerulein (50 µg/kg). Analgesia was provided by subcutaneous injection of buprenorphine (1 mg/kg) together with the first cerulein injection, and again with the 12th injection in animals culled later than the 12-hour time point. All animals were visually monitored throughout the procedure and removed from experiments if unexpectedly unwell. At the end of experiments, animals were culled by a method regulated by schedule 1 of the UK Animals (Scientific Procedures) Act 1986 at a time point stated in the chapter specific methods, but in general either one hour after the last cerulein injection (ie either 4 or 12 hours after induction of acute pancreatitis), or 24 hours after induction of acute pancreatitis.

Pre-treatments were given as stated in the text, but at least one hour before the first cerulein injection. Treatments were given as stated in the chapter specific methods, but in general together with the third cerulein injection.

Sample extraction from in vivo models

Details of sample collection and timings can be found in the chapter-specific methods. Where blood sampling was undertaken before induction of acute pancreatitis, this was from tail vein in temporarily immobilised animals according to standard procedures of the University of Liverpool Biomedical Sciences Unit. At the end of an experiment, blood was extracted using EDTA-coated syringes and sampling bottles via cardiac puncture. Approximately 600-800 µl blood was extracted per mouse. Blood was immediately spun using a table-top microcentrifuge at 1500 rpm for 10 minutes and plasma snap frozen on dry ice. Pancreas as well as both lungs (and other organs as detailed in specific methods) were extracted, washed in PBS and divided into three parts of similar volumes. Two pancreas segments were snap frozen on dry ice for MPO and trypsin activity analysis. The third segment was fixed in 4% formalin for at least 24 hours. The left lung was injected intra-bronchially with 4% formalin and fixed for at least 24 hours. The right lung was washed in PBS and snap frozen in dry ice for MPO activity assessment. Formalin-fixed specimens were submitted to The Royal Liverpool University Hospital Department of Histopathology for Paraffin embedding and 5 µm sectioning to NHS standards. Frozen samples were stored at -80 °C until further analysis.

2.3 *In vitro* experiments

Pancreatic acinar cell isolation

Murine pancreatic acinar cells were freshly isolated (Raraty, Ward et al. 2000; Gerasimenko, Gryshchenko et al. 2013; Huang, Booth et al. 2014) and cell death assays performed as previously prescribed, with minor alterations of

protocols (Zhang, Mizumoto et al. 1999; Huang, Cash et al. 2015). In brief, experimental animals were culled using an appropriate method regulated by schedule 1 of the UK Animals (Scientific Procedures) Act. All three lobes of the pancreas were surgically extracted from spleen to duodenum and washed in freshly made Na^+-HEPES buffer (containing 140 mM NaCL; 4.7 mM KCl; 10 mM HEPES; 1 mM $MgCl_2$; 10 mM glucose; 1.2 mM $CaCl_2$; adjusted to pH 7.3), referred to as HEPES buffer from this point. The pancreas was then injected with collagenase solution (200 units/ml HEPES) and incubated for between 15 and 20 minutes (depending on the batch of collagenase) in a shaking water bath at 37 °C in a 1.5 ml Eppendorf tube. Following incubation, the pancreas was washed in excess HEPES and shaken vigorously in a 15 ml Falcon tube. After one minute of sedimentation, the supernatant filtered through a 100 μm pore-size wet cell filter into a fresh 15 ml Falcon tube (cell suspension), and this cycle was repeated several times until the supernatant in the tube with the digested pancreatic tissue remained clear. The cell suspension was spun at 600 g for 60 seconds in a table top centrifuge, the pellet washed in HEPES buffer and spun again twice, before re-suspending the cell pellet in HEPES buffer (at about 10^6 cells/ml), ready for experimental use.

Cell death assays

For cell death assays, freshly isolated pancreatic acinar cells were suspended in HEPES in the presence of PI (2 μl) and seeded on a 96 well plate in a total volume of 200 μl per well. Signal was recorded every minute (Ex 540 nm/Em 620 nm) for 150 minutes using a BMG POLARstar Omega Microplate Reader (Imgen Technologies, New York, USA). Histones (50, 100 or 200 μg/ml) or digitonin (600 μM) was added after 30 minutes of establishing a stable

baseline. Percentage cell death in each experimental well was calculated as a proportion of maximum fluorescence in digitonin wells (Zhang, Mizumoto et al. 1999). Confocal images of isolated acinar cells were taken using a Zeiss LSM 510 or 710 (ZEISS Microscopy, Cambridge, UK) inverted confocal microscope with a 40x (air) or 63x (water) objective. Cells were seeded onto Poly-L-Lysine coated cover slips and placed in a perfusion chamber containing HEPES solution. Excitation lasers and emission spectra were selected to match fluorescent dyes used and minimise spectral overlap/background fluorescence. In general, FITC was excited using a 488 nm laser, collecting signal between 505 nm and 530 nm and PI excited using a 543 nm laser, collecting signal between 620 nm and 700 nm.

2.4 Reagents

Digitonin was from Calbiochem (Manchester, UK). BCA protein assay was from Thermo (Rockford, USA). Anti-histone H3 antibody was from Abcam (Rabbit monoclonal, 1:100 dilution; Abcam, Cambridge, UK), Calf-thymus histones, propidium iodide (PI), poly-D-glutamic acid (PGA), cerulein, acetic anhydride, protease inhibitors, phosphate-buffered saline (PBS) and other chemicals were from Sigma-Aldrich (Gilliangham, UK) of highest quality available. HEPES, Tris and Phosphate buffers were made fresh either on the day (HEPES) or within 4 weeks of use.

2.5 Sample processing

2.5.1 Biochemistry

For biochemical analysis of amylase, ALT and creatinine, plasma was diluted 1:4 in PBS (20 μl for amylase, 50 μl for ALT and creatinine) and submitted for

analysis to The Royal Liverpool University Hospital Department of Biochemistry for analysis to NHS standards.

2.5.2 Myeloperoxidase activity

Pancreatic and/or lung myeloperoxidase activity was determined as previously described (Dawra, Ku et al. 2008). Tissue samples were processed frozen. Samples were mechanically homogenised in phosphate buffer (100 mM, pH 7.4) spiked with protease inhibitors, before subjecting samples to two (lung) or three (pancreas) cycles of ultracentrifugation (16,000 x g, 15 minutes) and three freeze-thaw cycles in low pH phosphate buffer (100 mM, pH 5.4) containing 0.5% hexadecyltrimethyl ammonium bromide and 10mM EDTA.

Myeloperoxidase activity from the supernatant was measured in 3, 3', 5, 5'-tetramethylbenzidine solution by the addition of 0.01% H_2O_2 and recording the absorbance change at 655 nm over 3 minutes using a BMG POLARstar Omega Microplate Reader (Imgen Technologies, New York, USA).

Enzyme activity was reported per mg of protein as measured using a standard BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, UK) as per the manufacturer's instructions.

2.5.3 Trypsin activity

Samples were processed frozen similarly to previously published protocols (Nathan, Romac et al. 2005). Pancreas segments were homogenized in MOPS buffer (5 mmol/l MOPS; 1 mmol/l $MgSO_4$; 250 mmol/l sucrose; adjusted to pH 6.5) on ice before centrifugation at 1500 g for 10 minutes at 4 °C. 10 µl supernatant was added to a 96 well plate containing 50 µM Boc-Gln-Ala-Arg-MCA peptide substrate in 280 µl warmed Tris buffer (50 mmol/l Tris; 150 mmol/l

NaCl; 1 mmol/l $CaCl_2$; 0.01% BSA; 37 °C), collecting fluorescence signal at 440 nm with 380 nm excitation, once every minute for 6 minutes, using a BMG POLARstar Omega fluorescence microplate reader (Imgen Technologies, New York, USA). The rate of signal increase between 1 and 5 minutes was taken as a relative measure of trypsin activity and expressed as relative activity per mg protein as determined for each sample using a standard BCA protein assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, UK) as per the manufacturer's instructions.

2.5.4 Histone quantification

Plasma samples were obtained as above in EDTA tubes spiked with 0.1% UFH. Histone quantification was undertaken as previously described (Ou, Cheng et al. 2015), either in a blinded manner by Tingting Liu (see acknowledgement section), or as standard Western blot. In brief, plasma was subjected to SDS-PAGE using recombinant human histone H3 protein as standard and detected by Western blot (5% milk block for 1 hour, primary antibody incubation at 4 °C overnight, wash x3 with TBST, anti-rabbit IgG-HRP secondary antibody for 1 hour all except primary incubation at room temperature).

2.5.5 Histopathology

Histopathological slides were prepared and stained with Haematoxilin and Eosin by The Department of Histopathology at The Royal Liverpool University Hospital. Slide identifiers were covered, and histopathological scoring was performed on 10 random fields (x200 magnification) by two experienced, independent investigators blinded to experimental groups. Scores (0-4) were given for each of oedema, inflammatory cell infiltrate and acinar cell necrosis

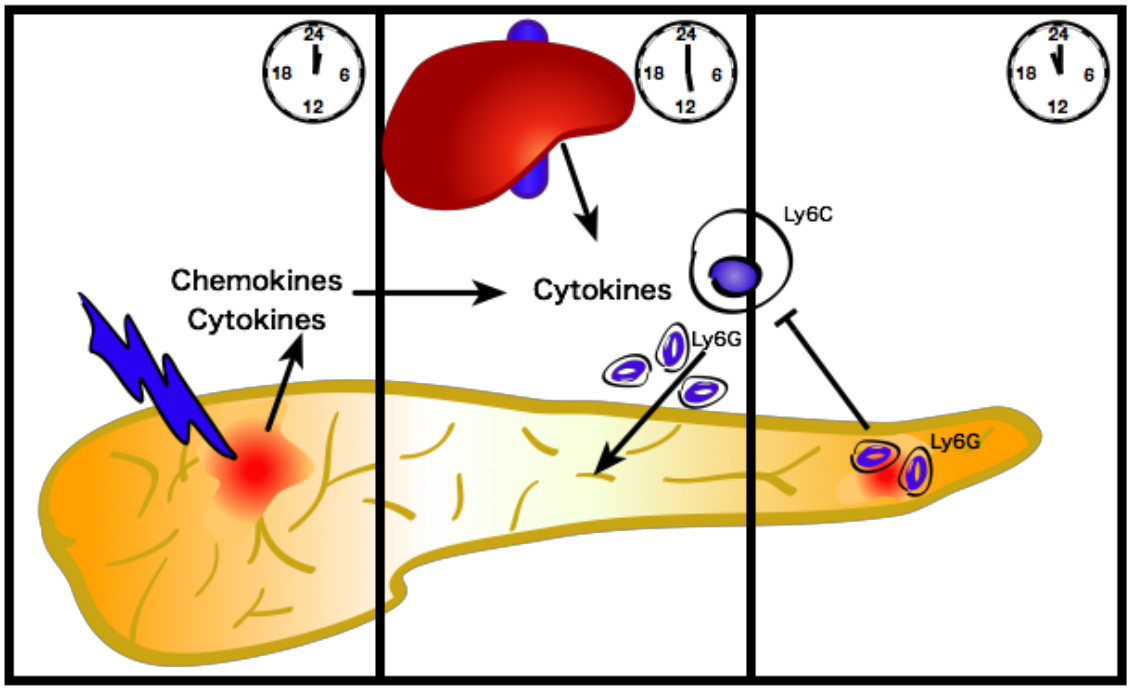
(Kusske, Rongione et al. 1996; Xu, Zhou et al. 2007) in a modification from the commonly used 3 point score (Van Laethem, Robberecht et al. 1996).

2.5.6 Data collection and analysis

Data was collected, stored and analysed using Prism 7.0c for Mac OS X. Data are presented as mean (+/- SEM) for parametric, or median (+/- IQR) for non-parametric data, and compared using Student's t-test (2-group comparison, parametric), ANOVA (3+-group comparison, parametric) or Wilcoxon signed-rank (2-group comparison, non-parametric) or Kruskal-Wallis (3+-group comparison, non-parametric) unless otherwise stated in the chapter specific methods. All graphical representations are generated using Prism 7.0c for Mac OS X, and all drawings were generated using Graphic for Mac v3.1.

3 The Inflammatory Sequence in Acute Pancreatitis

3.1 Visual abstract



1. Pancreatic injury results in release of chemokines (including CXCL-1 and CCL2).

2. The liver amplifies the signal of cytokines and chemokines. Inflammatory monocytes and neutrophils are recruited to the pancreas.

3. Infiltrating moncytes and neutrophils contribute to cellular injury, but inhibit release of further chemokines

3.2 Background

Acute pancreatitis is a disease with two distinct, yet interrelated components – pancreatic injury and sterile inflammation (Hoque, Malik et al. 2012; Habtezion 2015). Under physiological circumstances, neutrophils are absent in the pancreatic parenchyma (Sandoval, Gukovskaya et al. 1996). Acinar cell injury leads to the release of DAMPs and cytokines, which prime vascular endothelium to express adhesion molecules such as ICAM-1 and promotes recruitment of neutrophils and monocytes to the site of injury (Zaninovic, Gukovskaya et al. 2000; Habtezion and Algul 2016; Szatmary and Gukovsky 2016).

In human acute pancreatitis, a rapid rise of circulating neutrophils in relation to other leukocytes is associated with disease severity and organ failure (Zhang, Wu et al. 2016; Jeon and Park 2017) and MPO (Chooklin, Pereyaslov et al. 2009) and neutrophil elastase (Novovic, Andersen et al. 2013) activity correlates with disease severity. In experimental acute pancreatitis, neutrophil recruitment Is one of the earliest events in the disease happening within the first 2 hours following injury (Abdulla, Awla et al. 2011), peaking at 6 hours in a cerulein hyperstimulation model (Montecucco, Mach et al. 2014). Limiting this recruitment through genetic knock-out of ICAM-1 expression (Frossard, Saluja et al. 1999) or the neutrophil activating molecule NADPH Oxidase (Gukovskaya, Vaquero et al. 2002), inhibition of the CXCR2 chemokine receptor (Malla, Karrman Mardh et al. 2016) or by antibody-mediated depletion (Sandoval, Gukovskaya et al. 1996; Bhatia, Saluja et al. 1998; Pastor, Vonlaufen et al. 2006) improves biochemical and histopathological parameters of pancreas and lung injury.

Several mechanisms have been postulated, including activation of trypsinogen by neutrophil proteases (Abdulla, Awla et al. 2011; Awla, Abdulla et al. 2012; Tracy 2012), ROS-mediated injury (Montecucco, Mach et al. 2014) or NET-mediated tissue damage (Merza, Rahman et al. 2014; Korhonen, Dudeja et al. 2015). Inhibition of neutrophil elastase, which also effectively inhibits NETosis (Majewski, Majchrzak-Gorecka et al. 2016), has proved effective in preventing pancreatitis-associated lung and renal injury, but demonstrated only modest reductions in pancreatic injury (Wang, Tang et al. 2012; Cao and Liu 2013; Wang, Tang et al. 2013).

Some of the difference in the profound amelioration in pancreatic injury seen with neutrophil depletion versus functional inhibition might be explained by the methods used to achieve neutrophil depletion. Early studies used murine anti-neutrophil serum to achieve profound neutrophil depletion in rats (Sandoval, Gukovskaya et al. 1996; Bhatia, Saluja et al. 1998; Frossard, Saluja et al. 1999) and these all demonstrated significant improvement in pancreatic as well as lung injury. The traditional method of generation of anti-neutrophil serum results in polyclonal anti-serum, that likely depletes monocytes and platelets as well as neutrophils. Other studies used methotrexate (Fujimoto, Hosotani et al. 1997), anti-integrin (CD18) antibodies (Williams, Collins et al. 1990; Inoue, Nakao et al. 1995), or anti-GR-1 antibodies (Abdulla, Awla et al. 2011) to deplete neutrophils. These have effects other than neutrophil depletion, which are often not evident in the study methodologies. In particular, the GR-1 (but also CD18) depletion methods deplete pro-inflammatory monocytes as well as neutrophils (Egan, Sukhumavasi et al.

2008). This work attempts to detail the differential contributions of neutrophils and monocytes to the pathogenesis of cerulein pancreatitis.

3.3 Specific methods

3.3.1 Blood and tissue sampling

Blood for leukocyte phenotyping was collected fresh in EDTA bottles as per general methods. Splenocytes were generated by pressing freshly collected spleens through a 100 μm sterile cell filter, and isolated pancreatic acinar cells were generated by collagenase digestion as per the general methods but digesting for 20 minutes and filtering through a 75 μm sterile cell filter.

Blood for cytokine measurement was sampled in separate experiments, where animals were culled by overdose of anaesthetic and immediately upon cessation of circulation had 200 μl blood drawn into EDTA-laced 1ml insulin syringes from the hepatic portal vein, supra-hepatic vena cava and left ventricle. Blood was processed immediately as per general methods and plasma snap frozen on dry ice within 15 minutes of sample collection.

3.3.2 Reagents

10X RBC Lysis Buffer (Multi-species) and anti-mouse CD45 (FITC), anti-mouse CD11b (Pacific Blue), anti-mouse Ly6G RB6-8C5 (PE), anti-mouse Ly6C (APC) and anti-mouse F4/80 (eFluor 780) were from eBioscience (eBioscience Ltd, Hatfield, UK). BD FACS Flow sheath fluid and compensation beads (Becton, Dickinson and Company, Oxford, UK). Cytokine Bead Array – BD CBA Flex Set multiplex panel of beads: Mouse TNF; Mouse IL-6; Mouse MCP-1; Mouse IL-10; Mouse IFNγ; Mouse IL-1β; Mouse KC (all Becton, Dickinson and Company, Oxford, UK). Depletion antibodies InVivoMAb anti-mouse Ly6G (1A8) and

InVivoMAb anti-mouse Ly6C (Monts1), both Bxcell (2BScientific Ltd, Heyford, UK). Anti-CCR2 (MC-21) and control antibody (MC-68) were generously gifted by Prof Matthias Mack, University of Regensburg, Germany.

3.3.3 Leukocyte phenotyping

Antibodies were added to 50 μl whole blood, splenocytes or pancreas cell extract as per manufacturer's protocol (CD45/CD11b/L6G/Ly6C for whole blood, additional F4/80 for splenocytes and pancreas) and incubated in the dark for 30 minutes. Fluorescent panel compensations were generated using BD compensation beads according to the manufacturer's instructions in the standard manner. RBC lysis buffer was added for 10 minutes before centrifugation, wash and re-suspension in BD FACS Flow sheath fluid. Stained cells were immediately analysed using a BD Fortessa multi-colour flow cytometer (Becton, Dickinson and Company, Oxford, UK) with a 5-laser set-up. Data extraction and analysis was undertaken using Flow Jo for Windows. Figure 3-1 details the gating algorithms.

3.3.4 Cytokine measurement

Cytokine measurements were undertaken in thawed EDTA plasma at a 1:4 dilution as per manufacturer's instructions, similar to previous reports (Malla, Karrman Mardh et al. 2016). Beads were analysed using a BD Fortessa (Becton, Dickinson and Company, Oxford, UK) flow cytometer running FACSDIVA software v7, following manufacturer's manual set-up instructions. Protein standards were used to generate standard curves using Prism 7.0c for Mac using either linear or non-linear curve fits as appropriate (figure 3-2) and test concentrations extrapolated using these curves.

3.3.5 Leukocyte depletion

To ensure effective leukocyte depletion, animals were pre-treated with a single intra-peritoneal injection of relevant depletion antibody (500 μg anti-Ly6G, 500 μg anti-Ly6C or 75 μg anti-CCR2 antibody) 12 hours prior to induction of acute pancreatitis. Anti-Ly6G clone 1A8 was used for depletion of neutrophils, as this currently has the greatest neutrophil-specificity with minimal unwanted monocyte/platelet depletion (Daley, Thomay et al. 2008). 100 μl blood was sampled from tail veins of experimental animals prior to induction of acute pancreatitis and analysed flow cytometrically to confirm successful depletion. Blood was again taken at the time of tissue harvest and analysed to confirm persistent depletion of the relevant cell populations.

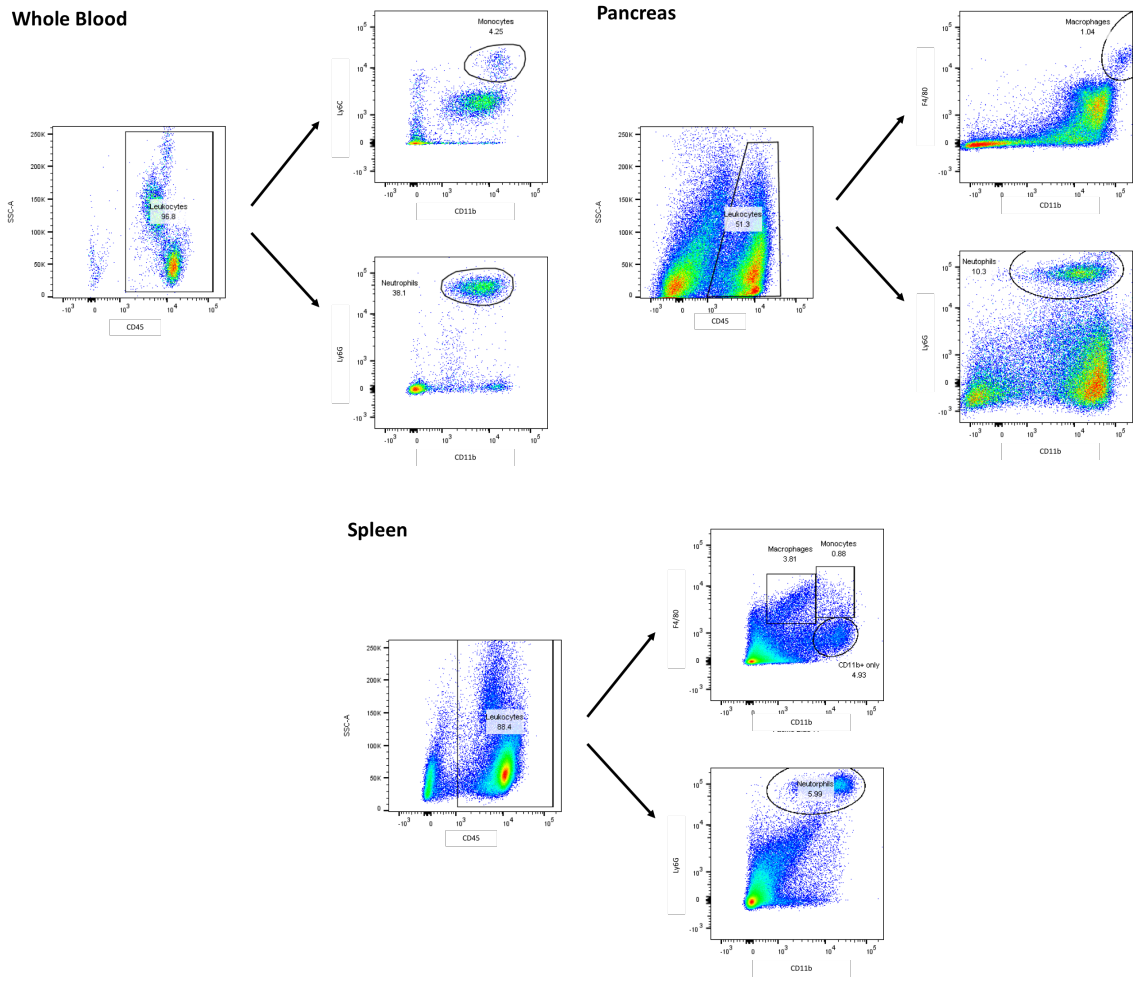


Figure 3-1 Gating strategies in the identification of monocyte, macrophage and neutrophil populations in murine whole blood, pancreas and spleen. The initial whole-cell population (left) selects the leukocyte population gating on CD45 positivity, whereas monocytes (Ly6C+/CD11b+; top right) and neutrophils (Ly6G+/CD11b+; bottom right) are selected from the leukocyte population. Cell viabilities >95% for all cell types.

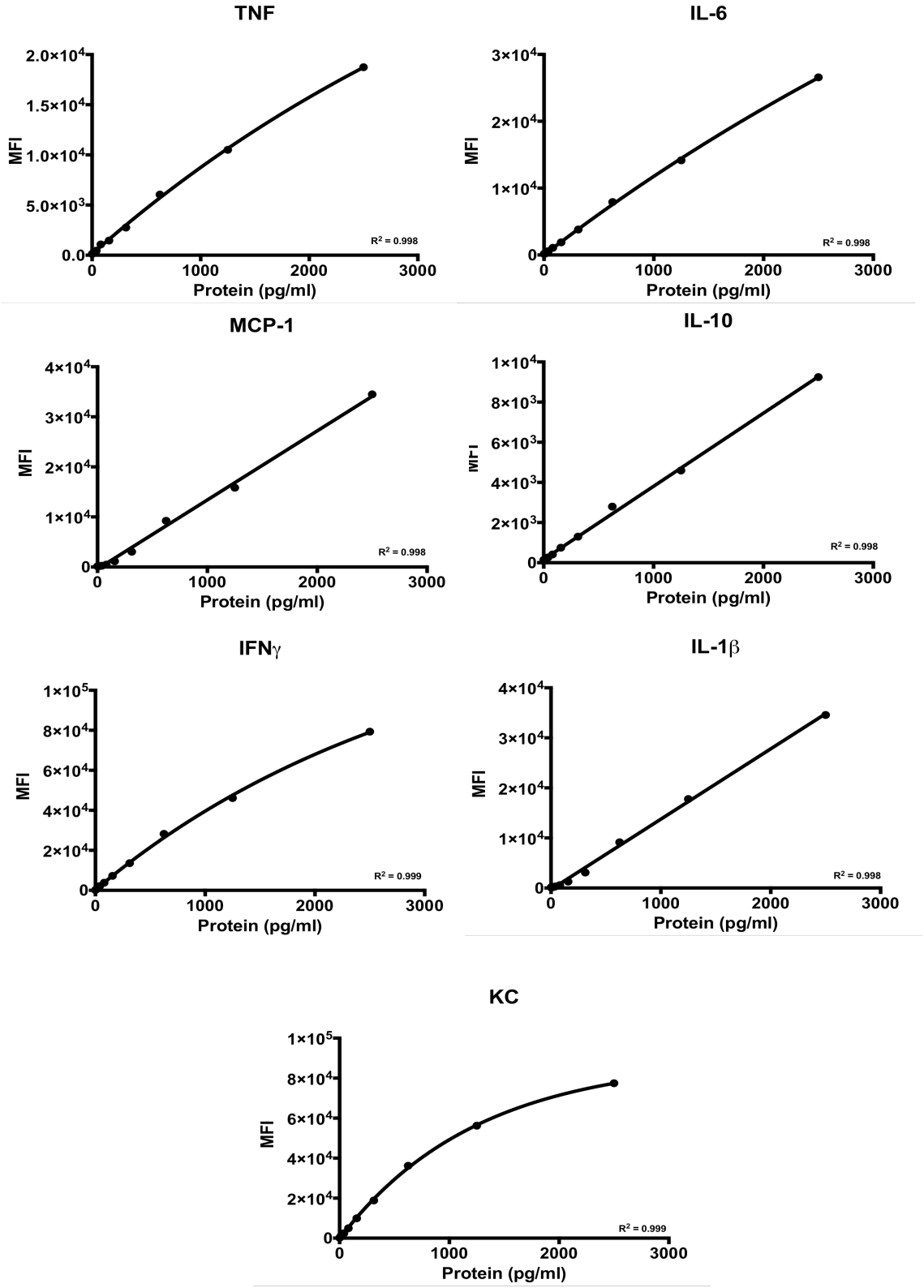


Figure 3-2 Standard curves of mean cytokine bead array fluorescence generated from recombinant protein standards with associated linear or non-linear regression curves used for extrapolation of cytokine concentrations. R^2 consistently > 0.99 for all measured cytokines.

3.4 Chemokines attract myeloid cells to the inflamed pancreas while the liver amplifies systemic cytokine signals

In order to identify the source of chemokines and cytokines released in the early phases of acute pancreatitis blood was sampled from the portal vein and thoracic vena cava in mice, immediately following induction of acute pancreatitis with 12 hourly injections of cerulein and 12 hours thereafter. A panel of 6 pro-inflammatory cytokines was selected based on known relevance to leukocyte migration and previous investigations in the context of experimental pancreatitis. The final panel measured concentrations of IL-1β, TNF, CCL2, CXCL-1, IL-6 and IFNγ using a cytokine bead assay (CBA) analysed by flow cytometry. Except for IFNγ, which was consistently below the detection threshold of the test, all cytokines were included for analysis.

Figure 3-3 details chemokine levels 12- and 24-hours following induction of experimental acute pancreatitis with 12 hourly injections of cerulein. As expected, chemokine concentrations were highest in portal vein blood indicating the pancreas as likely source. Chemokine levels in central venous blood of pancreatitis animals were indistinguishable from those in control animals after 24 h (CCL2, p=0.4326; CXCL2, p=0.7144), suggesting either that pancreatic chemokine production/release had ceased by 24 h or that there was a negative feedback system between chemokines and their target cells to limit the overall inflammatory response.

Figure 3-4 demonstrates the differences observed with cytokines. All three cytokines measured were present in similar, or higher in the case of TNF, concentrations in portal and central venous circulation. As central venous blood is a mixture of portal and systemic blood, the finding that there is no

significant dilution of cytokine concentrations suggests that the liver is a major producer of these cytokines in systemic circulation, and in the case of TNF is the main source of the cytokine in systemic circulation.

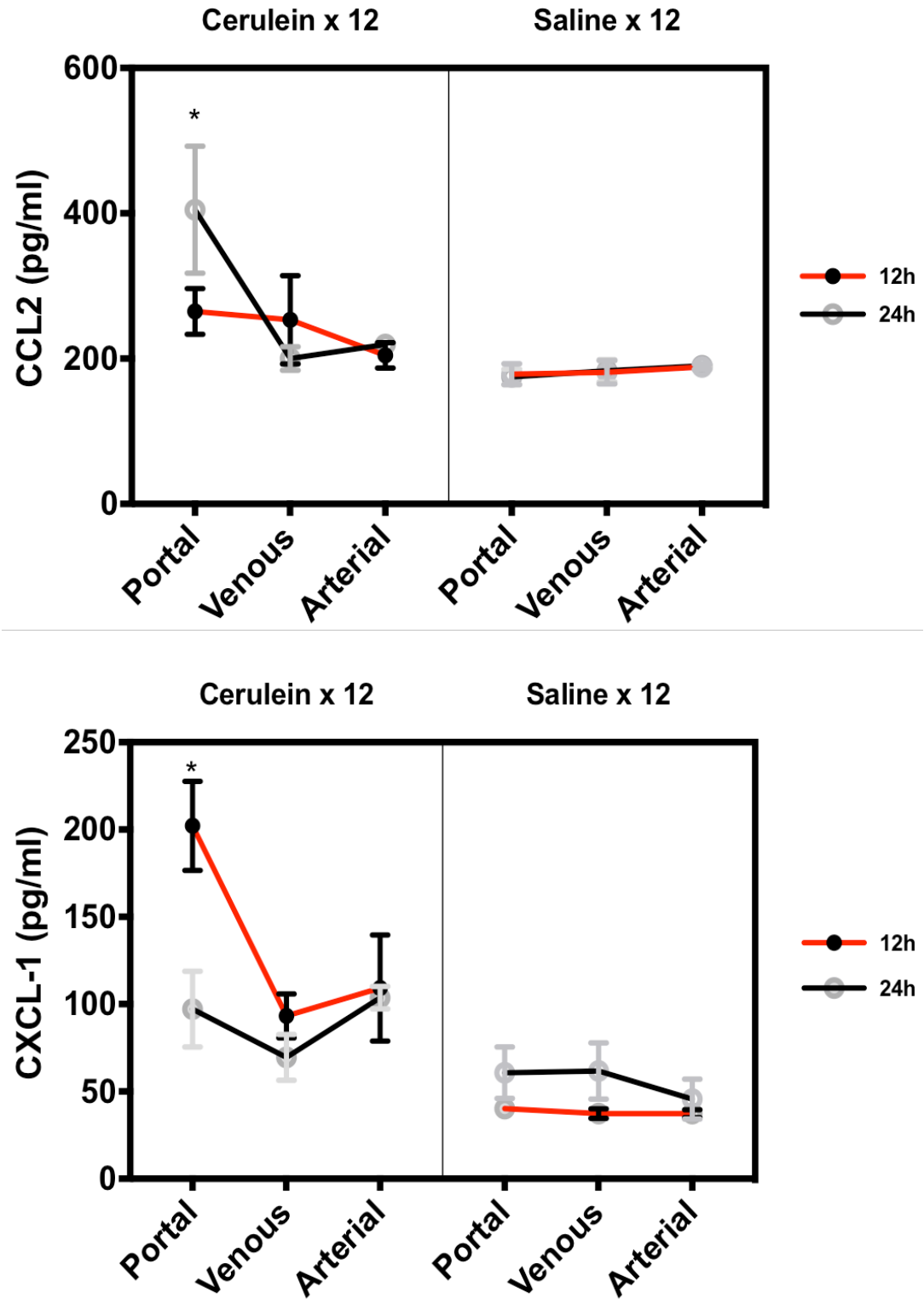


Figure 3-3 Chemokines CCL2 (MCP-1) and CXCL-1 (KC) measured in blood from portal vein, thoracic vena cava and left ventricle from mice with acute pancreatitis induced by 12 hourly cerulein injections or saline controls. Samples were taken one and 13 hours after the final cerulein injection. Comparisons were made by 2-way ANOVA and multiple t-tests using Benjamini, Kriegler, Yekutieli false-discovery method. * denotes $p<0.05$ between anatomical locations and timepoints; $n \geq 6$.

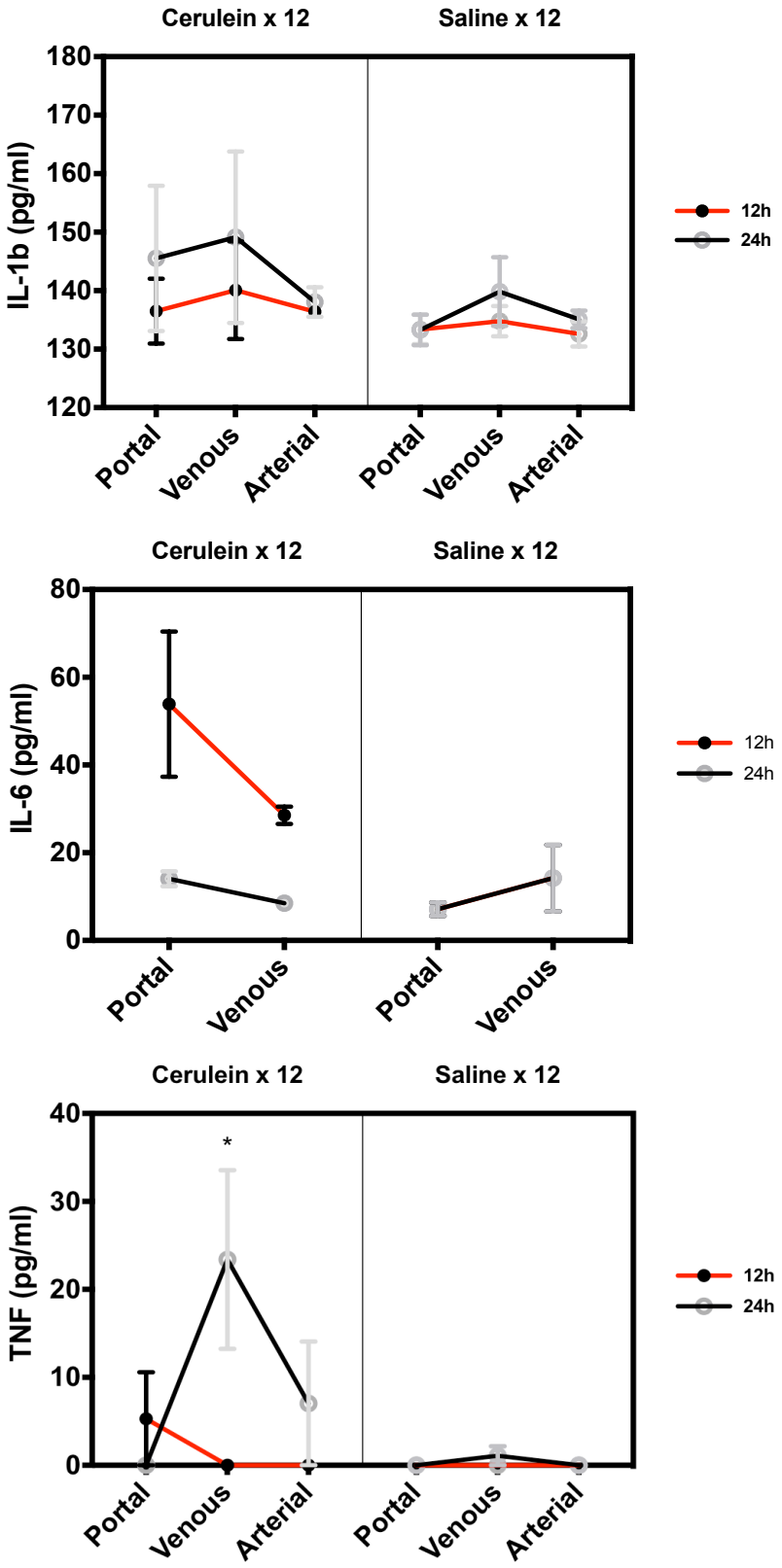


Figure 3-4 Cytokines IL-1β and TNF measured in blood from portal vein, thoracic vena cava and left ventricle from mice with acute pancreatitis induced by 12 hourly cerulein injections or saline controls. Samples were taken one and 13 hours after the final cerulein injection. IL-6 measurements were taken from portal and systemic venous blood only. Comparisons were made by 2-way ANOVA and multiple t-tests using Benjamini, Kriegler, Yekutieli false-discovery method. * denotes $p<0.05$ between anatomical locations and timepoints; $n \geq 6$.

3.5 Inflammatory monocytes and neutrophils are recruited to the pancreas and dampen further chemokine release

In inflammation research, particularly in acute pancreatitis, an assumption is frequently that changes in peripheral blood reflect changes at organ level. This is a pragmatic view when investigating human pancreatitis as deep tissue samples are impractical, however it is an assumption that can be tested in experimental models.

Figure 3-5 demonstrates changes in numbers of monocytes (Ly6C positive cells) and neutrophils (Ly6G positive cells) as a proportion of leukocytes (CD45 positive cells) or total cells following induction of acute pancreatitis or in saline controls in blood, spleen and pancreas. The proportion of neutrophils rises in blood following induction of pancreatitis, which is mirrored by a rise of neutrophil numbers in the pancreas and spleen. Monocytes, however, reduce in number in blood, but increase early in the pancreas and after 24 h in the spleen, possibly representing the extravasation of inflammatory cells into their target organ and lymphatic system. Neutrophils may be recruited in such large numbers from cellular stores that no similar drop is seen in blood.

To assess roles of classical monocytes (Ly6C positive), inflammatory monocytes (CCR2 positive) and neutrophils (Ly6G positive), these cell types were individually depleted using monoclonal antibodies. Each monoclonal antibody effectively depletes its respective cell type, without significant effects on the other (Figure 3-6). Following induction of acute pancreatitis, monocyte depletion has no effect on blood neutrophil levels, but neutrophil depletion doubles blood monocytes ($p=0.0028$), suggesting neutrophils suppress monocyte expansion via a negative feedback mechanism. On a

cytokine level (Figure 3-7), neutrophil depletion drastically increases levels of the chemokine KC in circulation, which hints at a possible mechanism for this feedback loop. Depletion of inflammatory monocytes, but not classical monocytes, increases levels of circulating TNF and CCL2 (MCP-1), suggesting an inflammatory monocyte-specific feedback mechanism.

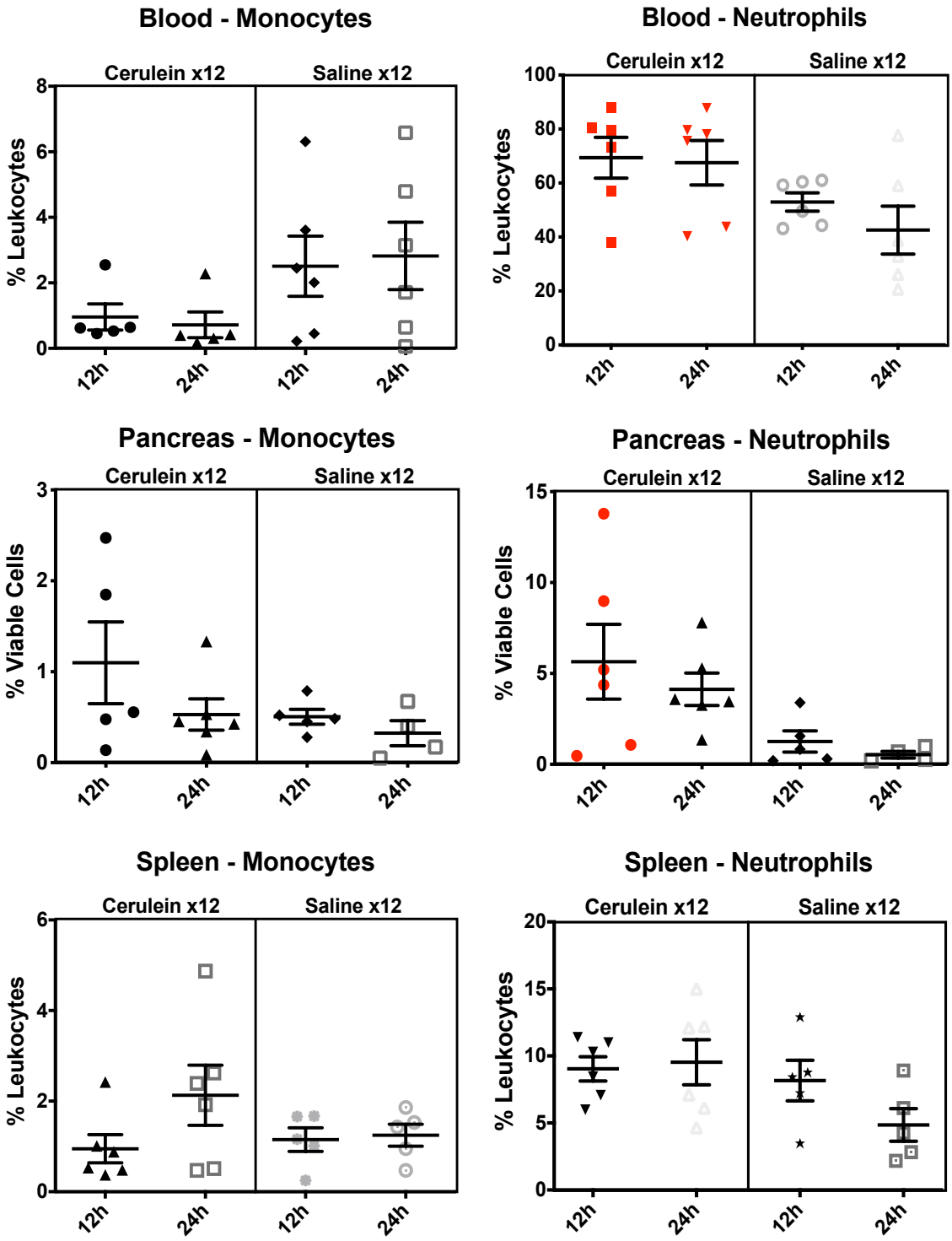


Figure 3-5 Monocytes (Ly6C+) and Neutrophils (Ly6G+) as a proportion of leukocytes (CD45+) or total viable cells measured with multi-colour flow cytometry. Data points are from whole blood, pancreas (collagenase digestion) and spleen (homogenate) from CD-1 Swiss mice 12 and 24h following induction of acute pancreatitis with 12 hourly injections of cerulein (50µg/kg, i.p.). Comparisons are made to saline control by ANOVA, where red colour of the data set indicates $p<0.05$; $n \geq 5$.

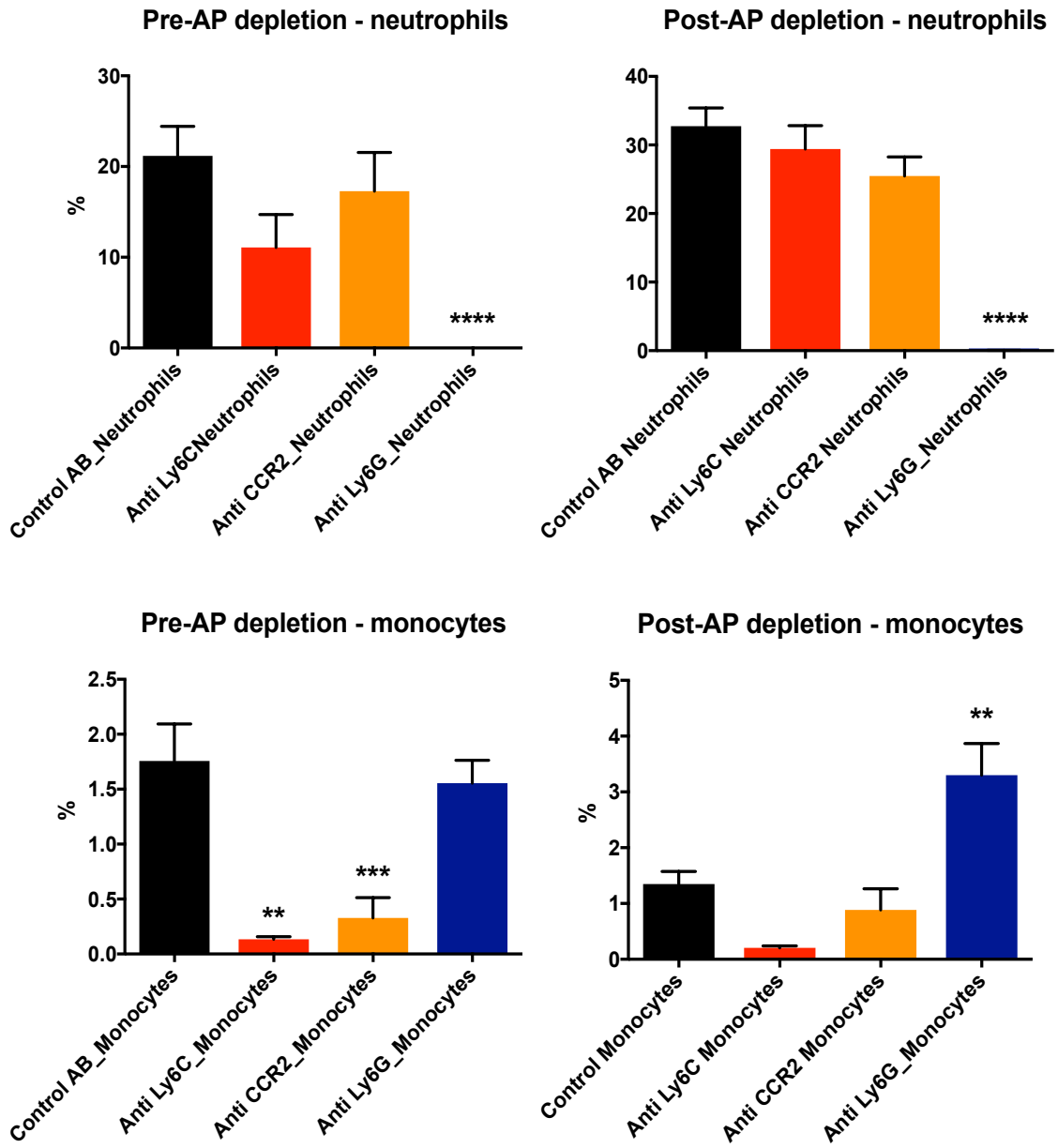


Figure 3-6 Effect of neutrophil or monocyte depletion on the other cell-type in blood. Blood samples were taken from tail vein of CD-1 Swiss mice prior to induction of acute pancreatitis, and from cardiac puncture 24h following 12 hourly injections of cerulein (50µg/kg, i.p.). Cell numbers refer to event numbers (Ly6G – neutrophils, Ly6C – monocytes) measured by multi-colour flow cytometry as a proportion of total leukocyte (CD45+) numbers. All comparisons are to the control group by ANOVA, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.000$; $n \geq 6$.

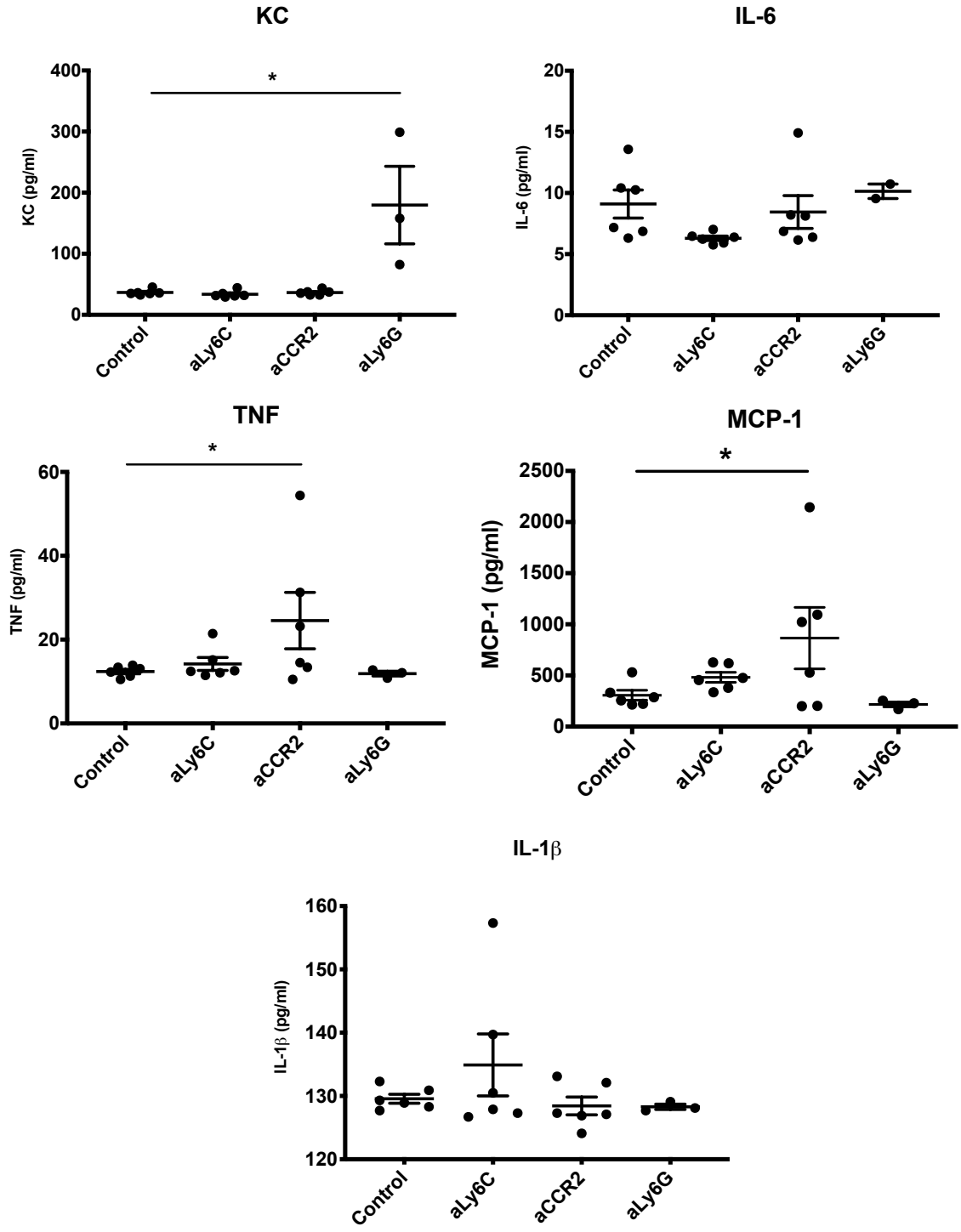


Figure 3-7 Plasma cytokine levels measured by cytokine bead assay from CD-1 Swiss mice 24 hours after induction of acute pancreatitis with 12 hourly injections of cerulein (50μg/kg, i.p.) and following depletion of classical monocytes (Ly6C), inflammatory monocytes (CCR2) and neutrophils (Ly6G) with a single injection of the relevant monoclonal antibody 12h prior to induction of pancreatitis. All comparisons are to the control group by ANOVA, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; $n \geq 6$.

3.6 Monocytes have a greater role in pancreatic injury, whereas neutrophils make a greater contribution to lung injury.

To assess the roles of the three myeloid cell types (classical monocytes, inflammatory monocytes and neutrophils) on the pathogenesis of acute pancreatitis, the effect of cell depletion on pancreatic and lung myeloperoxidase activity, trypsin activity, plasma amylase levels (Figure 3-8) and pancreatic histopathology was measured (Figure 3-9). Depletion of classical monocytes led to a statistically significant reduction in pancreatic (but not lung) MPO as well as plasma amylase levels. Inflammatory monocyte depletion reduced lung MPO and pancreatic trypsin activity levels as well as plasma amylase, and neutrophil depletion reduced pancreatic and lung MPO as well as pancreatic trypsin activity levels. Only monocyte depletion demonstrated a statistically significant improvement in pancreatic histopathology, with the most notable improvement being a reduction in pancreatic necrosis.

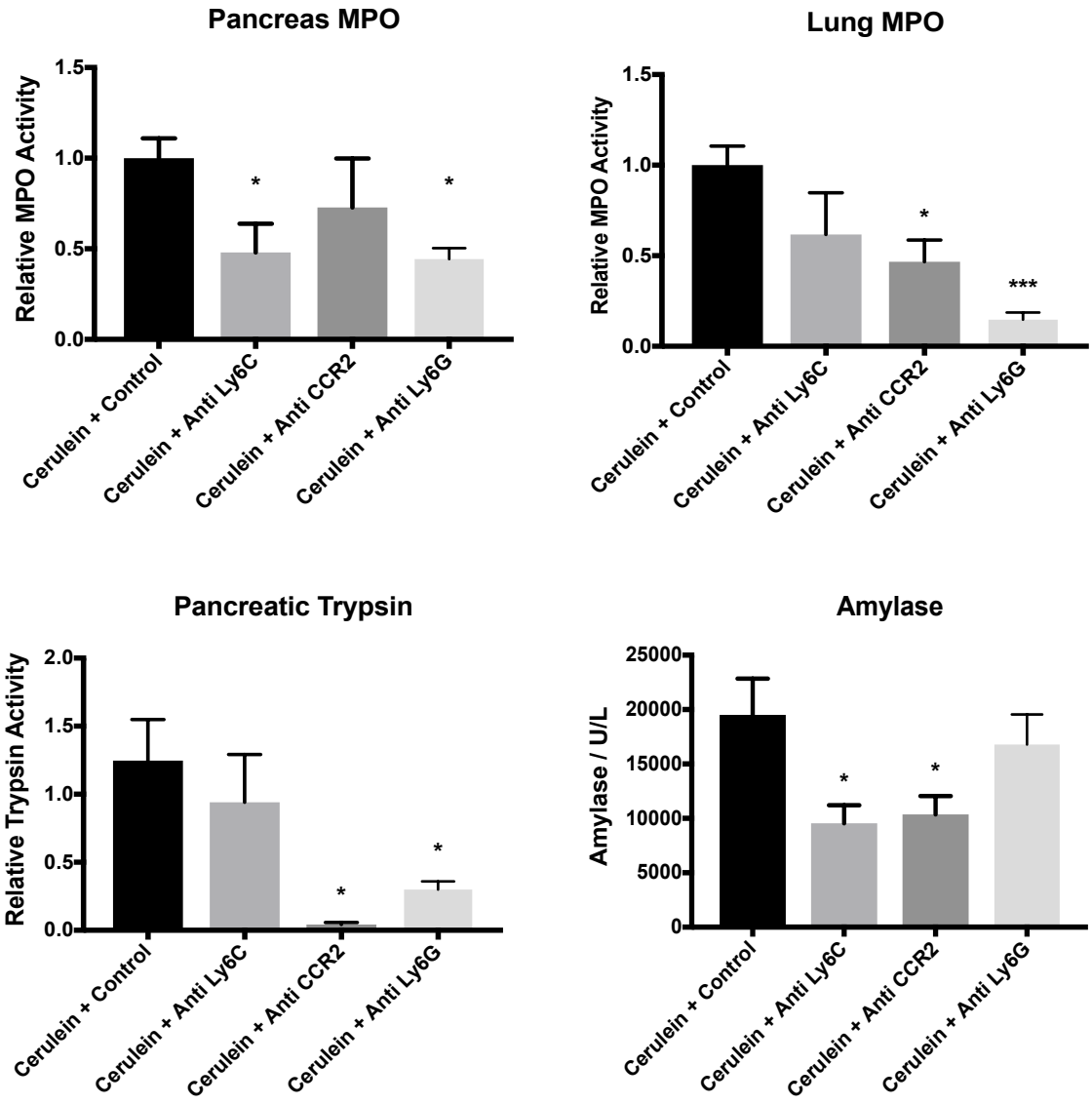


Figure 3-8 Biochemical parameters (pancreas and lung MPO activity, pancreatic trypsin activity and plasma amylase) from organ homogenates and plasma of CD-1 Swiss mice after induction of acute pancreatitis with 12 hourly injections of cerulein (50μg/kg, i.p.) and depletion of classical monocytes (Ly6C), inflammatory monocytes (CCR2) and neutrophils (Ly6G) using specific monoclonal antibodies. All comparisons are to the control group by ANOVA, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; $n \geq 6$.

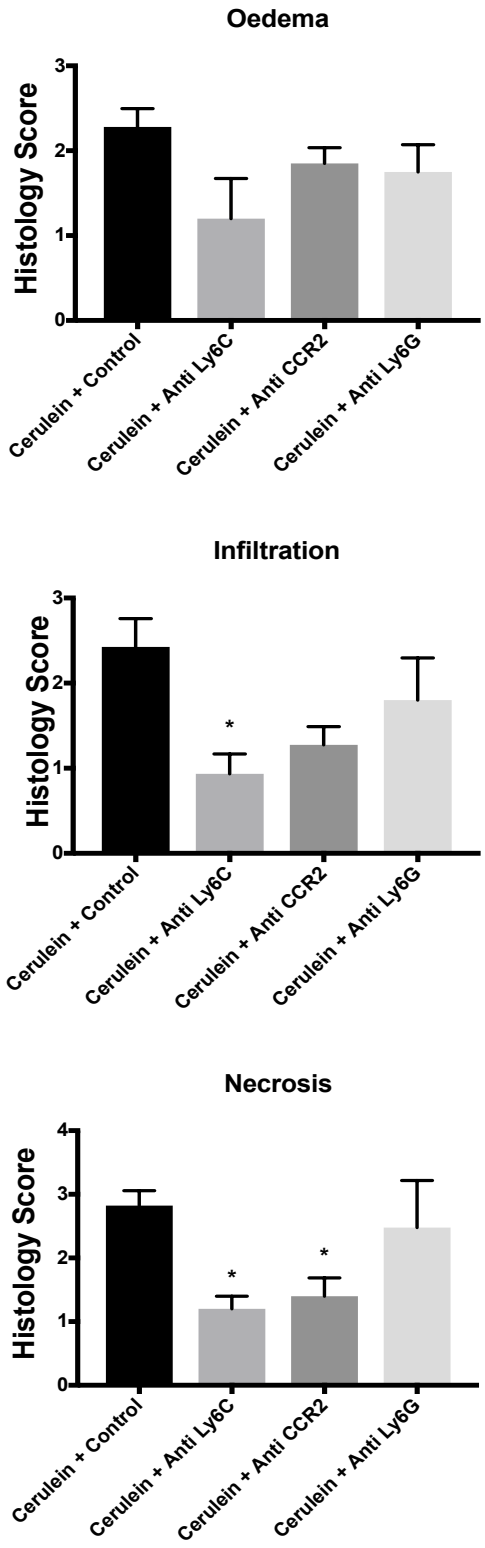


Figure 3-9 Histopathological scores from pancreata of CD-1 Swiss mice pre-treated with depletion of classical monocytes (Ly6C), inflammatory monocytes (CCR2) and neutrophils (Ly6G) using specific monoclonal antibodies. All comparisons are to the control group by ANOVA, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; $n \geq 6$.

3.7 Discussion

The combined actions of classical monocytes, inflammatory monocytes and neutrophils in acute pancreatitis secondary to cerulein hyperstimulation are such as to exacerbate pancreatic injury in the short term (ie within 24 hours). The actions of each individual cell type, however, appear to be more nuanced. Chemokines including CXCL-1 (KC) and CCL2 (MCP-1) are released into the portal vein, resulting in recruitment of monocytes and neutrophils to the pancreas. This or other signals lead to cells within the liver, possibly Kupffer cells (Gloor, Blinman et al. 2000; Folch-Puy 2007); hepatic stellate cells (Stefanovic, Brenner et al. 2005); or peritoneal macrophages (Rehermann 2016), to produce and release cytokines (TNF and IL-1β) into the systemic circulation. Although generally thought of as redundant signalling, depletion of individual cell types leads to the increase in concentrations of individual cytokines (Neutrophils – KC, inflammatory monocytes – CCL2 and TNF, classical monocytes – IL-1β), raising the possibility of cell-specific feedback pathways. Furthermore, the pattern of injury in experimental acute pancreatitis is specific to each cell type, with neutrophils contributing predominantly to protease activation and lung injury, but monocytes being primarily responsible for pancreatic cellular injury and the rise in systemic amylase.

These data are of interest, as they hint at a more nuanced pathogenesis than previously assumed, which opens new avenues for clinical exploitation. However, it needs to be regarded with due caution. CCL2 (but not CCR2) deficient animals, for example, show a blunted response to cerulein-induced acute pancreatitis (Frossard, Lenglet et al. 2011). However, animals deficient

in CCR2, i.e. those unable to recruit inflammatory monocytes, exhibit greater neutrophil infiltration and hyperglycaemia following chronic cerulein administration (Nakamura, Kanai et al. 2013). Data from our study demonstrates the cytokine feedback mechanisms as well as the controls that monocytes place on neutrophils and vice-versa. Blockade of a single factor can, therefore, lead to an unexpected increase in another. The varied responses measured in response to different cytokine inhibitors may reflect the different roles of different cell types at different stages in the pathogenesis of pancreatitis, and a cell such as the neutrophil that is injurious in the short term and in its local micro-environment may actually be acting to limit wider organ injury. A similar phenomenon has been observed in sepsis, where neutrophils lead to intravascular thrombosis in hepatic sinusoids to limit the spread of micro-organisms (Clark, Ma et al. 2007).

The use of a single mouse model of acute pancreatitis – cerulein hyperstimulation – is a clear limitation of this study. However, it is a reliable and extremely reproducible model, and avoids the need for laparotomy which in itself leads to tissue injury and inflammation. While 12 hourly injections of cerulein produce comparable degrees of pancreatic injury (Huang, Cane et al. 2017) and systemic DAMP release (Ou, Cheng et al. 2015) as retrograde perfusion of the bile duct with bile acids, it does not produce a significant systemic inflammatory response, which is reflected by the relatively low levels of cytokines measured (Szatmary and Gukovsky 2016). The method of depletion used led to an effective reduction in the cell type in question that lasted for the duration of the experiment in the case of neutrophils and classical monocytes. Total levels of monocytes had partially recovered by the

end of the experiment when depleting inflammatory monocytes alone. This could indicate that there is a pool of inactive monocytes that only express CCR2 when activated, making antibody-mediated depletion in an untreated animal ineffective. In any case, it suggests that the effects of CCR2 depletion could have been even more pronounced if it had been achieved more effectively.

Sampling from multiple sources (blood, spleen and pancreas), demonstrates very well how changes in blood do not always reflect changes in the target tissue, although the changes in the target organ are consistent and predictable. This is likely to be highly relevant for human disease. Moreover, it has been demonstrated that measurements of leukocyte populations vary greatly within the same animal depending on sampling site (Nemzek, Bolgos et al. 2001), presumably due to changes to endothelial adhesion molecules. This can further limit the comparability of experimental studies, or indeed human studies using peripheral and central blood interchangeably.

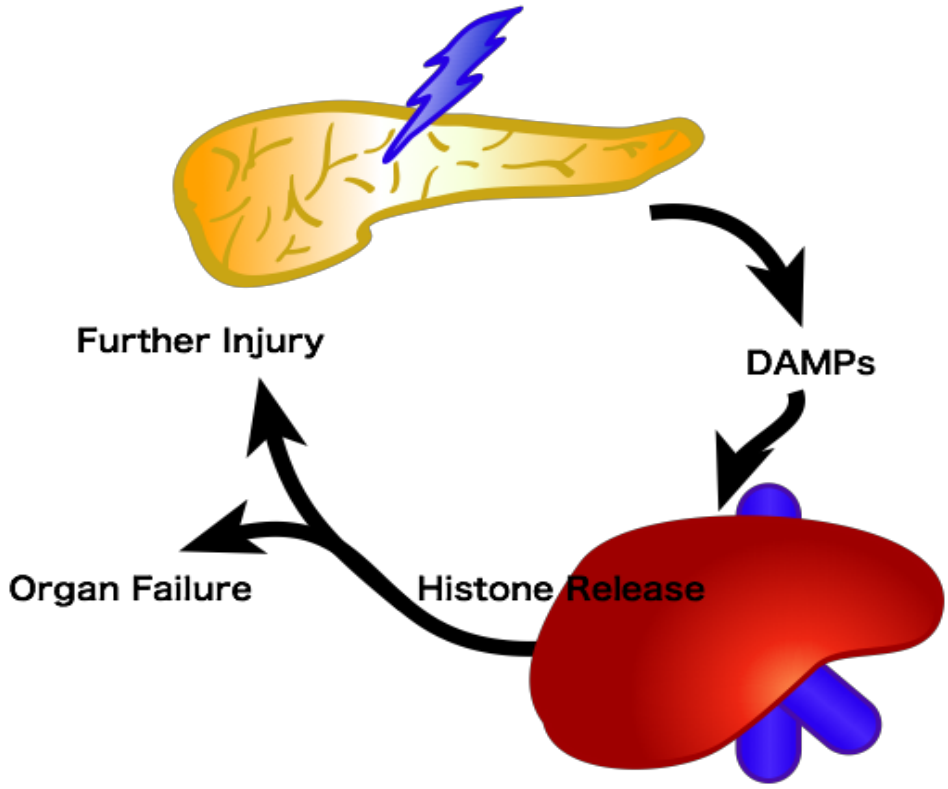
3.8 Summary

Pancreatic injury results in the release of chemokines that lead to the recruitment of neutrophils as well as inflammatory and classical monocytes to the pancreas, where the resulting cellular inflammation exacerbates the initial injury. These early responder cells then dampen further cytokine release and promote resolution of inflammation in complex interactions between themselves and through cytokine signalling. A better understanding of these interactions could help to design strategies to improve monitoring and influence the pathogenesis of acute pancreatitis in the future. Lessons about

the importance of sampling site and timing should be applied to human trial designs.

4 Extracellular Histones Contribute to Disease Severity in Acute Pancreatitis

4.1 Visual abstract



4.2 Background

Pancreatic acinar cell injury leading to the release of pro-inflammatory cytokines and recruitment of immune cells is a well investigated process (Habtezion 2015; Habtezion and Algul 2016; Gukovskaya, Gukovsky et al. 2017) and the cellular events are described in greater details still in the previous chapter. Acute pancreatitis is itself associated with systemic release of histones, the amount correlating with disease severity in animal models (Ou, Cheng et al. 2015), however the precise source of histones is not known. Necrotic acinar cells seem a likely candidate, as specific neutrophil depletion had little effect on pancreatic injury as seen in the previous chapter.

Extracellular histones also contribute to lung injury when injected systemically (Saffarzadeh, Juenemann et al. 2012; Zhang, Guan et al. 2016), leading to pulmonary vascular endothelial activation and neutrophil trapping. As well as lung injury, extracellular histones can induce cardiac, hepatic and renal injury (Kawai, Kotani et al. 2016) in mice, a phenomenon reminiscent of the multi-organ failure seen in clinical severe acute pancreatitis. One of the functions of C-reactive protein (CRP), the best current biomarker in the prediction of severity of acute pancreatitis, is to bind and detoxify circulating histones (Abrams, Zhang et al. 2013), suggesting a rise in CRP might be in response to a rise in circulating histones.

This chapter aims to investigate the utility of measuring circulating histones as nucleosomes in human acute pancreatitis, determine the source of extracellular histones in circulation during acute pancreatitis using a cerulein model and evaluate the effects of exogenous histones in the pathogenesis of the disease.

4.3 Specific methods

4.3.1 Human samples

Human plasma samples from the NIHR Liverpool Pancreas Acute Pancreatitis Biobank from 50 randomly selected patients was analysed (see general methods for a detailed sample description). Nucleosome levels were measured using the Roche Cell Death Detection ELISA with minor modification from the manufacturer's instruction as previously described (Abrams, Zhang et al. 2013). Absorbance values were recorded using a BMG POLARstar Omega Microplate Reader (Imgen Technologies, New York, USA) and reported as relative to the lowest recorded value. Disease severity (final severity classified according to the revised Atlanta classification (Banks, Bollen et al. 2013)) and CRP at 24 and 48 hours after admission was also collected and used to calculate ROC curves using Prism 7.0c for Mac.

4.3.2 In vivo experiments

Both mild (4 injection) and severe (12 injection) models of cerulein acute pancreatitis were used (see general methods). FITC-tagged histones (20 mg/kg) were administered via the tail vein immediately following the last cerulein injection using the 4-injection model only. Animals were sacrificed 6 hours following administration of the first cerulein injection. Organs were harvested, washed in PBS and briefly dried on sterile gauze. Organs were imaged using an IVIS Spectrum preclinical imaging system (Perkin Elmer, Waltham, MA, USA) utilizing the epifluorescence function collecting signal for 8 seconds. In selected experiments, calf-thymus histones (20 mg/kg) or PBS

(200 µl) were administered in the same way as above. Animals were sacrificed and tissues harvested for further analysis 12 hours after the first cerulein injection. For portal, central venous and arterial blood sampling animals received an overdose of pentobarbital, abdominal and thoracic cavities where opened and plasma samples taken into EDTA syringes containing 1% v/v heparin from portal vein, thoracic inferior vena cava and left ventricle. Histone quantification was performed by Western blot and densitometry performed in ImageJ (Schneider, Rasband et al. 2012).

4.3.3 Reagents

For some experiments histones were conjugated with fluorescein isothiocyanate (FITC) and passed through an ion exchange column to remove excess dye according to established procedures (Fattahi, Grailer et al. 2015). FITC-conjugated histones were generated and gifted by Dr Simon Abrams (see acknowledgements). Cell Death Detection ELISA was from Roche (Roche Diagnostics Ltd, Burgess Hill, UK).

4.4 Circulating nucleosome levels correlate with disease severity in human acute pancreatitis

Nucleosomes, i.e.: DNA-associated histones, are easier to measure than histones themselves, as nucleosomes can be measured using a simple sandwich ELISA, whereas histones are measured using qualitative or quantitative Western blotting. Therefore, levels of circulating nucleosomes were measured in plasma from 50 patients (mild and/or moderate, n=36; severe, n=14) with acute pancreatitis selected at random and obtained from the NIHR acute pancreatitis biobank. Levels were then correlated with data on disease severity and C-reactive protein concentrations.

Levels of circulating nucleosomes were significantly higher in severe, compared to mild/moderate disease (Figure 4.1, P<0.0001). ROC curve analysis revealed 0.87 accuracy (Figure 4.1, P=0.001) in discriminating between mild/moderate and severe acute pancreatitis on admission, compared to an accuracy of 0.72 (P=0.049) and 0.82 (P=0.0046) when using CRP measured within 24 and 48 hours of admission respectively. This highlights the potential clinical utility of measuring circulating nucleosome levels in predicting persistent organ failure in acute pancreatitis. These findings were confirmed by direct measurement of histones by quantitative Western blot (Figure 4.1), leading to an expansion of the dataset and subsequent publication (Liu, Huang et al. 2017).

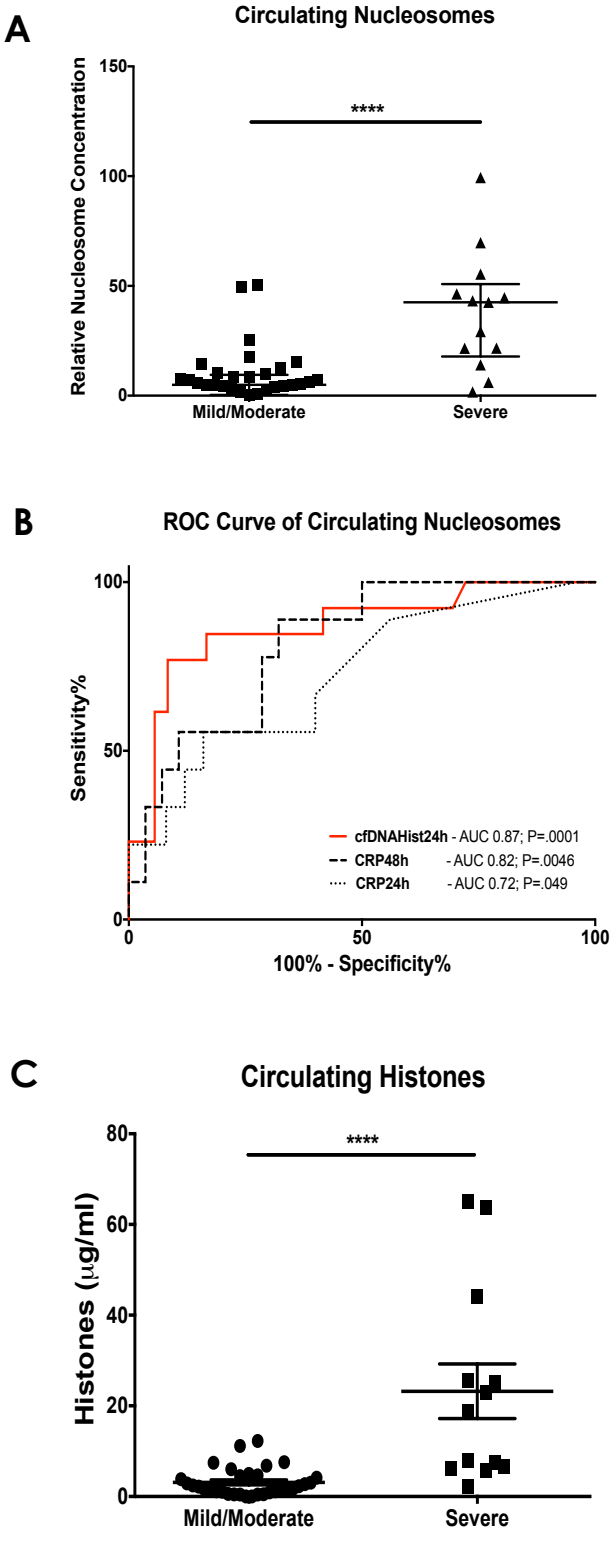


Figure 4-1 A) Levels of circulating nucleosomes relative to each other in plasma from patients with mild, moderate or severe acute pancreatitis according to the revised Atlanta classification of Acute Pancreatitis. **B)** Receiver operator characteristic of circulating nucleosomes on admission, as well as CRP on admission and at 48 hours in predicting severe disease at any time during admission. **C)** Quantitative Western blot of circulating histones in patients with mild, moderate or severe acute pancreatitis. Comparisons between two groups were done using the Mann-Whitney U test, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$; n = 50.

4.5 The liver is the primary source of circulating histones in cerulein pancreatitis

It is easy to assume equal distribution of soluble factors in circulation. However, as demonstrated in the previous chapter, soluble molecules released in response to injury that carry a signalling function can vary greatly in concentration depending on the location of production/release. The hypothesis with histones in the context of acute pancreatitis was that histones are either released passively as a consequence of acinar cell necrosis, or actively as a result of NETosis. To test this hypothesis, plasma was sampled from murine portal vein and vena cava blood following induction of acute pancreatitis with 12 hourly i.p. injections of 50 µg/kg cerulein. Surprisingly, there was a 20-fold greater concentration of histone H3 in post-hepatic central venous blood compared to post-pancreatic portal blood or arterial (cardiac) blood (Figure 4.2; $P<0.05$).

To investigate this phenomenon further, systemic levels of the hepatocellular enzyme alanine aminotransferase (ALT) were measured and livers of pancreatitis and control animals subjected to histopathological analysis. Plasma levels of ALT were elevated 4-fold in cerulein pancreatitis compared to controls, and while liver histopathology did not demonstrate any hepato-cellular necrosis, it did reveal vacuolisation (consistent with cellular stress) in oxygen-poor zone 3 (Figure 4-3).

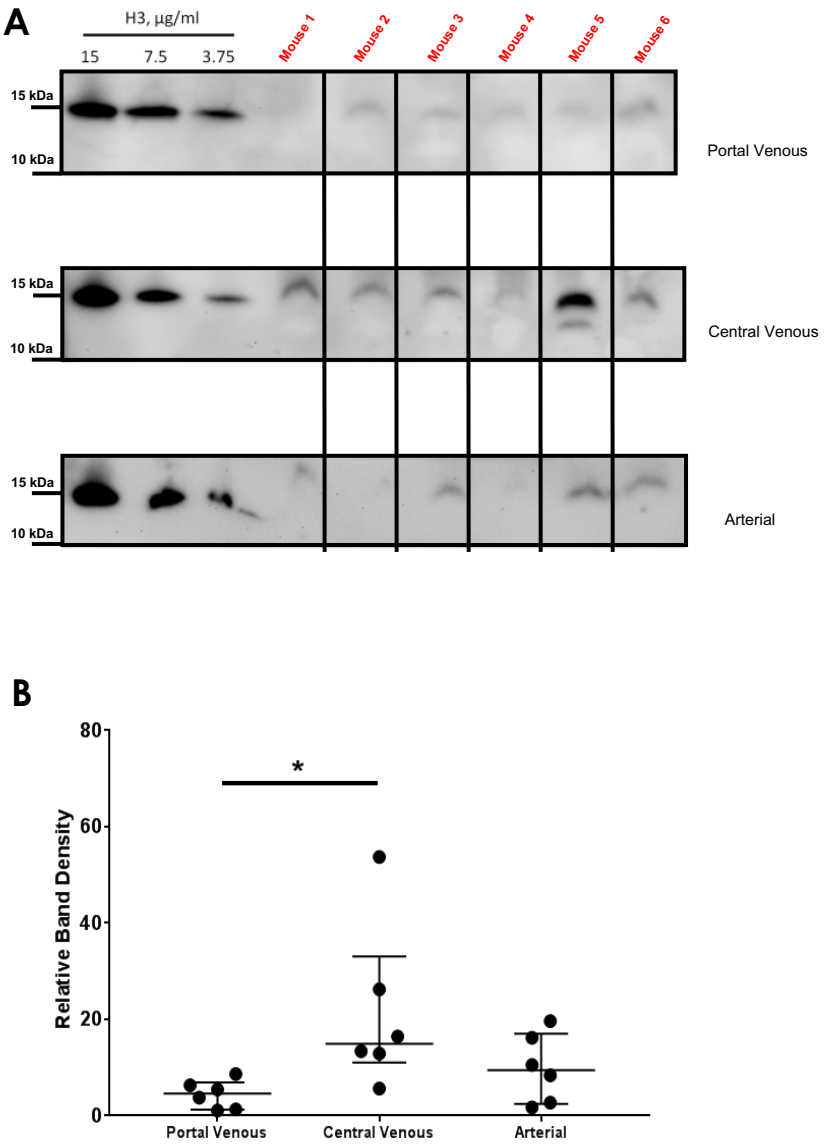


Figure 4-2 A) Western blot of histones in murine plasma after induction of acute pancreatitis with 12 i.p. injections of 50 µg/kg cerulein, sampled from portal vein, vena cava and left ventricle in the same animal. Parallel lines link bands from the same animal. **B)** Relative density of histone H3 bands from different sample locations. All comparisons are to each other group by ANOVA, where * denotes $p<0.05$; $n = 6$.

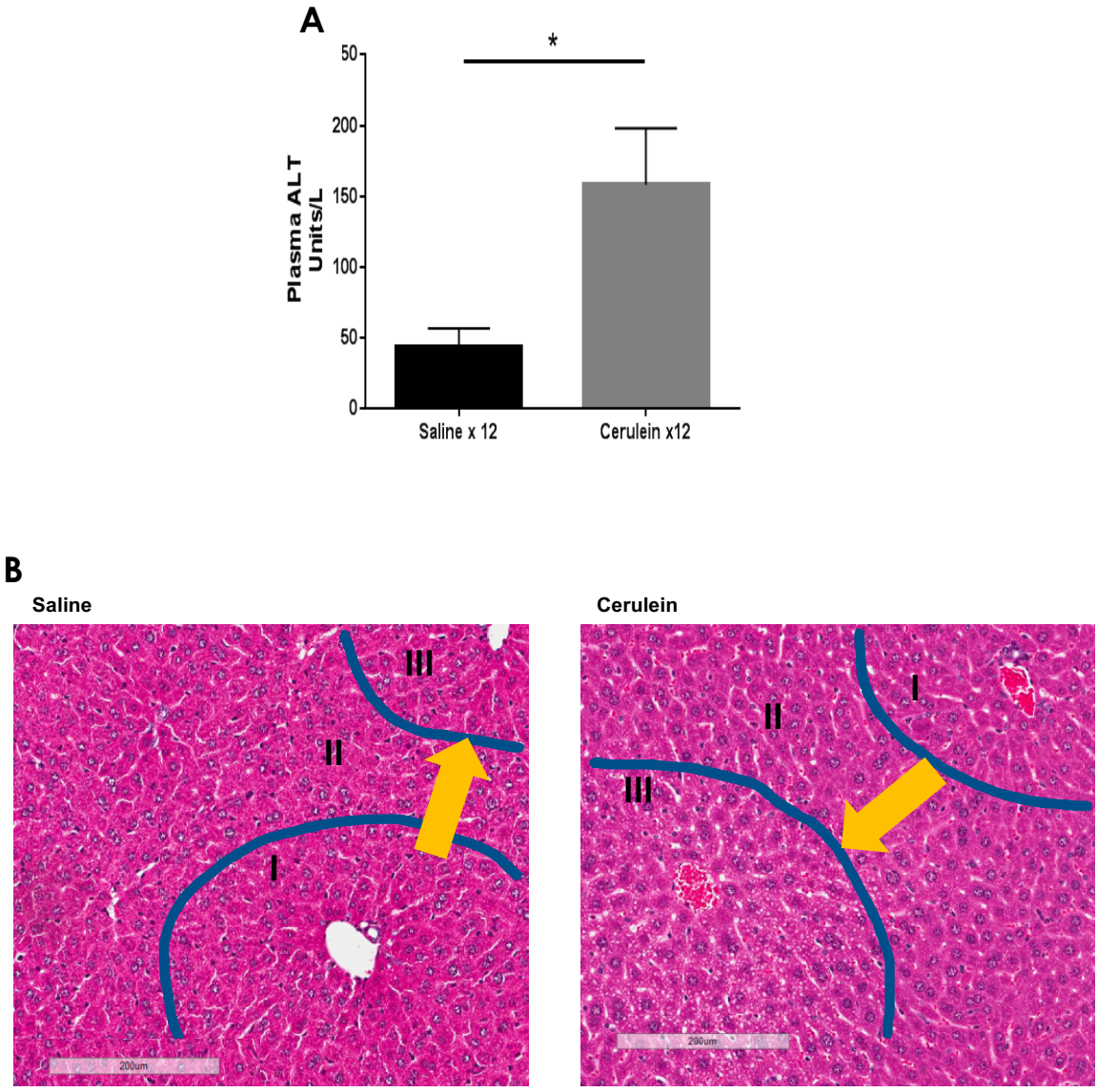


Figure 4-3 A) Plasma ALT levels in mice with acute pancreatitis induced by 12x i.p. injections of 50 µg/kg cerulein and saline controls. **B)** Histopathological slide of liver (x20 magnification) stained with Haematoxilin and Eosin, denoting perfusion zones I-III and direction of blood flow (yellow arrow). Comparisons are made using Student's t-test, where * denotes $p<0.05$; $n \geq 6$.

4.6 Systemically administered histones exacerbate pancreatic injury in mild oedematous experimental pancreatitis

Having established a correlation between histones in circulation and severity of acute pancreatitis in patients, as previously in cerulein pancreatitis (Ou, Cheng et al. 2015), and identified an extra-pancreatic source for histones, the possibility of a causal relationship between circulating histones and severity of pancreatitis presents itself. To test causality, a milder form of experimental acute pancreatitis was induced using 4x hourly i.p. injections of cerulein together with a sub-lethal dose (20 mg/kg) of histones i.v (tail vein) together with the 4th injection of cerulein. Serum biochemistry and pancreas histology was evaluated 12 hours after induction of pancreatitis, allowing comparison with the severe model.

Histone administration alone did not raise serum amylase or pancreatic MPO activity levels above control, but co-administration of histones in the context of mild cerulein-induced pancreatitis markedly increased pancreatic MPO activity as well as inflammatory cell infiltrates and acinar cell necrosis beyond control, as confirmed by blinded histopathological scores (Figure 4-3).

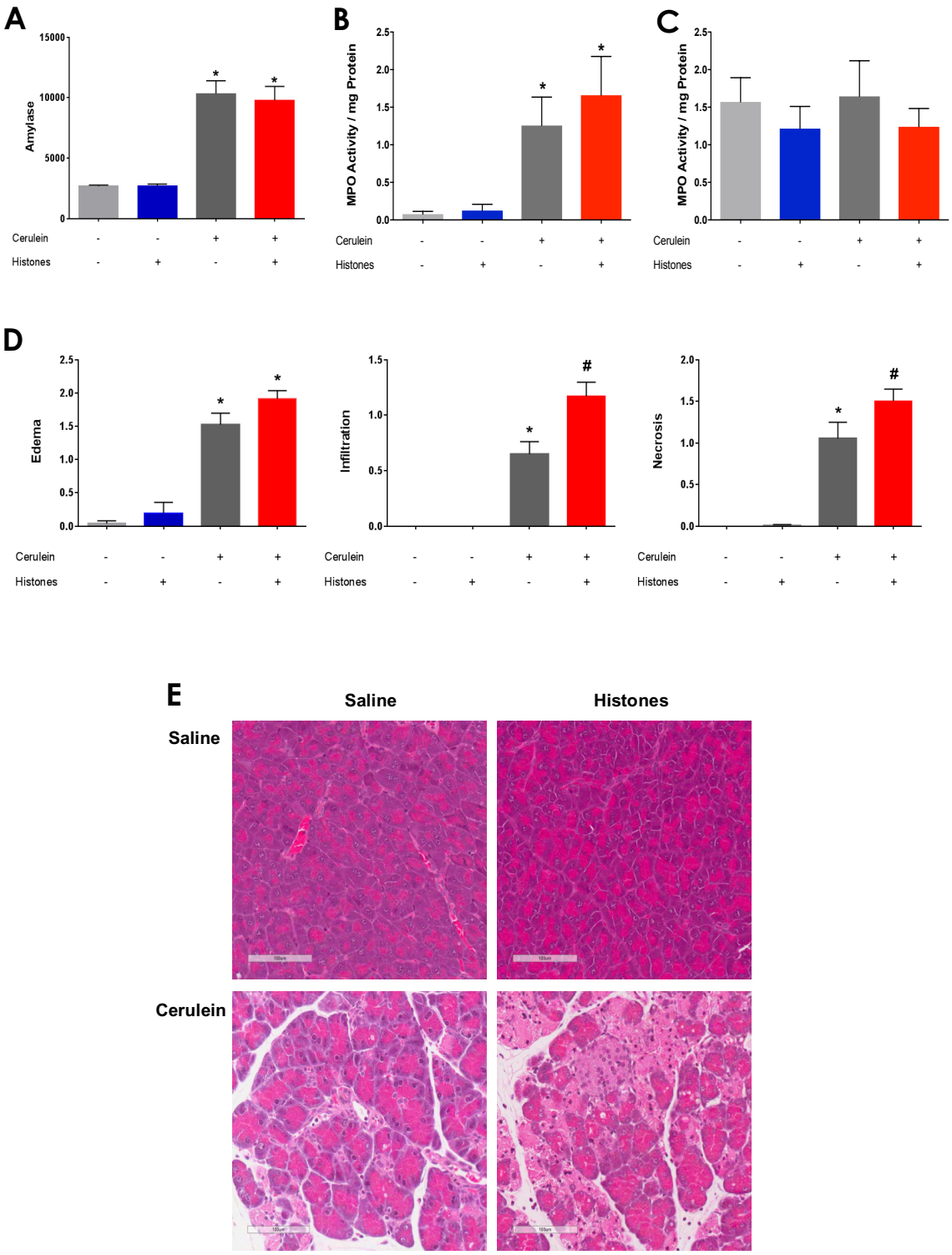


Figure 4-4 A) Serum amylase concentration, **B)** pancreatic and **C)** lung myeloperoxidase activity and **D)** blinded histopathological assessment of the pancreas (oedema, infiltration and necrosis) in experimental acute pancreatitis induced by 4x hourly i.p. injections of 50 µg/kg cerulein or saline controls, both with and without co-administration of 20 mg/kg i.v. calf thymus histones. **E)** Representative micrograph of histologic appearance of pancreata of treated animals. All comparisons by ANOVA, where * denotes a difference of $p<0.05$ to control and # denotes a difference of $p<0.05$ to cerulein; $n \geq 6$.

4.7 Systemically administered histones concentrate within an inflamed pancreas

The results above suggest administering histones systemically can increase necrosis and inflammation within the pancreas itself. The mechanism for this is not clear. Previous work, using FITC-labelled histones injected into the tail vein and then measuring fluorescence in organ homogenate suggested that systemically injected histones concentrated within the lung (Fattahi, Grailer et al. 2015). The results above do not demonstrate elevation of lung MPO following intravenous administration of histones, making significant pulmonary injury as an indirect cause of exacerbated pancreatic necrosis (e.g. through hypoxia) unlikely.

To identify the site of action of intravenously injected histones, FITC-labelled histones were injected into the tail veins of C57Bl6/J mice, similarly after 4x hourly i.p. injections of 50 µg/kg cerulein or saline. Animals were sacrificed 6 hours after the first i.p injection and internal organs (heart, lung, liver, pancreas, spleen, kidneys and bladder) were extracted and imaged using an IVIS Spectrum epifluorescence chamber. The only detectable FITC signal was in pancreata of cerulein-treated mice (Figure 4-5). There was marked autofluorescence in gall bladders and bladders of mice, which was present irrespective of FITC administration. Critically, there was no signal in pancreata of saline-treated animals, or when injecting unconjugated FITC (20 g/kg) as a control, indicating concentration of histones within the inflamed pancreas and not hyperaemia, or an exudative mechanism. There was no detectable signal in heart, lungs, liver, kidneys or spleen in cerulein or control animals.

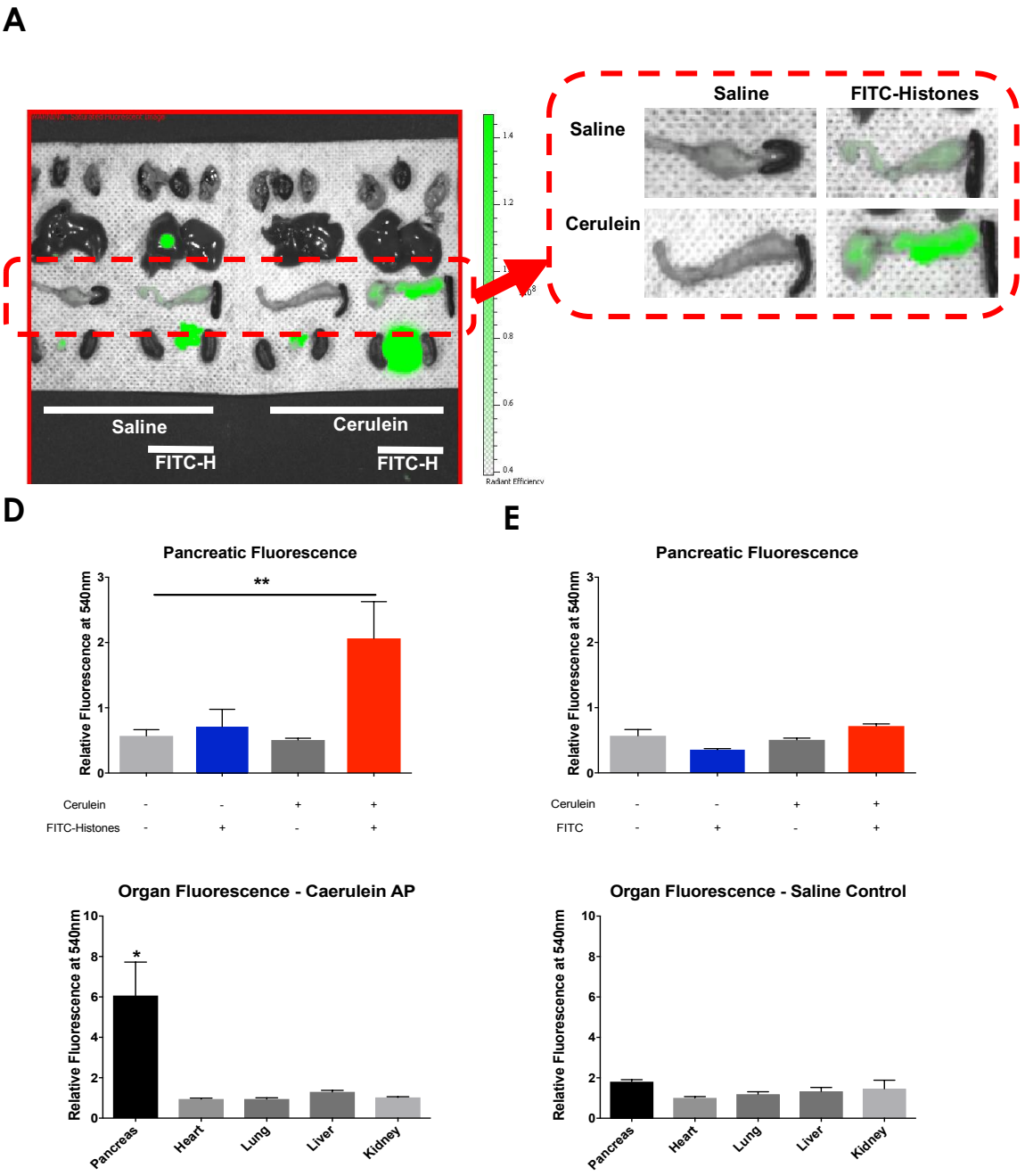


Figure 4-5 A) Epifluorescent image of murine organs following i.v. injection of FITC-labelled histones in cerulein AP or saline controls; inset is close-up view of pancreas and spleen. Fluorescence intensity in pancreata of **B)** cerulein or **C)** saline treated animals with or without administration of FITC-labelled histones. Fluorescence intensity of all extracted organs (except bladder) in **D)** cerulein AP or **E)** saline controls. All comparisons are by ANOVA (parametric data) or Kruskall-Wallace test (non-parametric). * denotes $p<0.05$, and ** $p<0.01$; $n \geq 5$.

4.8 Discussion

Extracellular histones are known mediators of acute inflammation (Allam, Kumar et al. 2014; Kawai, Kotani et al. 2016). As nuclear proteins, a rise in plasma concentration would be expected in a disease marked by tissue necrosis such as acute pancreatitis, especially when taking active release by infiltrating inflammatory cells into account (Merza, Hartman et al. 2015). Indeed, the data presented here confirms the correlation between circulating nucleosomes and histones already reported in experimental models of AP (Ou, Cheng et al. 2015) as well as in patients (Penttila, Rouhiainen et al. 2016). It goes further to confirm the same correlations holds for circulating histone H3 and that this increase is detectable within 24 hours of admission to hospital, while the absolute concentration is more accurate in predicting disease severity than the current best biomarker - CRP measured at 48 hours.

Current understanding leads to the hypothesis that this rise in circulating histones in acute pancreatitis is due to a combination of pancreatic cellular necrosis and intra-pancreatic extracellular trap release, however this would mean the highest measurable histone concentration in any given animal with acute pancreatitis should be in the first common drainage channel – the portal vein. The data presented here clearly demonstrate peak histone concentrations in the post-hepatic vena cava, concluding that the liver is the predominant source of histones in circulation. While there is a concomitant rise in ALT and structural hepatocyte damage in poorly oxygenated zone 3, it is worth noting that the role of ALT in human acute pancreatitis is less clear, as the two commonest causes of human acute pancreatitis (gallstones and ethanol) can independently affect ALT levels. Apoptosis of lymphocytes (Bell

and Morrison 1991) in systemic circulation has also been postulated as primary cause of the rise in circulating histones in AP (Ou, Cheng et al. 2015) or sepsis (Liu, Ni et al. 2013). The relatively low histone concentrations in arterial blood, however, adds further support to the liver as primary source in our model and indeed allows us to hypothesise that the pulmonary circulation acts as a filter for circulating histones. It is possible that rather than hepatocyte injury, resident Kupffer cells or peritoneal macrophages, recruited to the liver in inflammation (Niu, Bian et al. 2016; Rehermann 2016), contribute to the release of histones in response to portal vein DAMPs as previously shown in vitro (Chen, Fu et al. 2015). This interpretation would be supported by data showing reduced liver injury in experimental AP following Kupffer cell depletion (Zhang, Dang et al. 2010) as well as reduced lung injury seen in AP with Kupffer cell inhibition (Liu, Cui et al. 2006). As one of the earlier descriptions of the role of hepatic NETs was to limit systemic spread of micro-organisms in sepsis (McDonald, Urrutia et al. 2012) and bacterial translocation resulting from intestinal barrier failure is a hallmark of human and experimental acute pancreatitis (Rychter, van Minnen et al. 2009; Koh, Jeon et al. 2012), portal sepsis may be the principle determinant of hepatic histone release. This hypothesis would provide a mechanistic link between pancreatitis severity and hepatic NET/histone release, and a potential explanation how pancreatic infection could contribute to disease severity (Petrov, Shanbhag et al. 2010). The use of only a single experimental model of acute pancreatitis is an obvious limitation when making conclusions about the source of histones in acute pancreatitis.

Irrespective of the primary source of circulating histones, the data here presented confirm a causal relationship between circulating histones and

disease severity in acute pancreatitis. Systemic injection of histones not only leads to a concentration of histones within the inflamed pancreas but exacerbates organ injury. The apparent contradiction with the only previous bio-distribution study of circulating histones (Fattahi, Grailer et al. 2015), which used a very similar methodology, can be explained by the differences in disease models. Fattahi and colleagues used an experimental sepsis model and found histone concentration within the lung. They also used a higher dose of histones (45 mg/kg), much closer to the lethal dose of 75 mg/kg, which has been shown to lead to rapid death within hours from pulmonary oedema and alveolar haemorrhage (Abrams, Zhang et al. 2013). Fattahi's finding is also consistent with the finding that histones only concentrate within an injured and inflamed pancreas. The fact that un-conjugated FITC does not lead to a similar increase in fluorescence indicates that this is an active process, rather than leakage into the tissues as oedema. It is possible that extravasation of net-positively charged histones is facilitated by the exposure of net-negatively charged proteoglycans such as heparin sulphate. Given that histones themselves exert antimicrobial activity and that infection of necrosis significantly increases mortality in necrotizing pancreatitis (Petrov, Shanbhag et al. 2010; Hackert and Buchler 2016; Werge, Novovic et al. 2016), this mechanism may even offer some survival benefit at the cost of exacerbating disease in the short term.

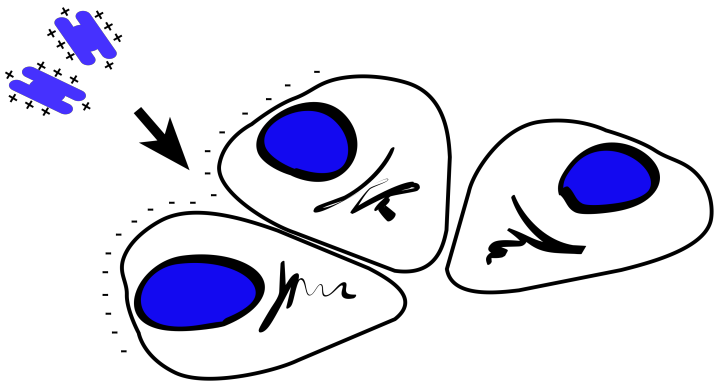
4.9 Summary

Collectively, these data demonstrate circulating histones are important early mediators of pancreatitis severity and implicate the liver as the primary source in circulation; circulating histones concentrate within the inflamed pancreas

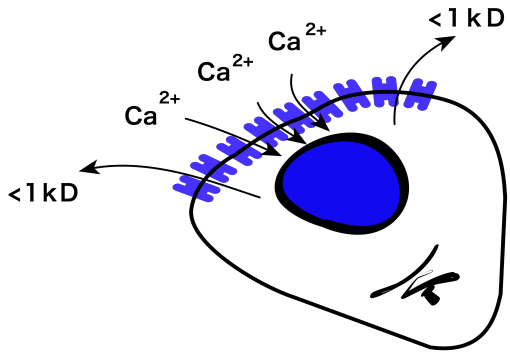
and actively contribute to pancreatic acinar cell necrosis and pancreatic inflammation.

5 Histones are Toxic to Pancreatic Acinar Cells

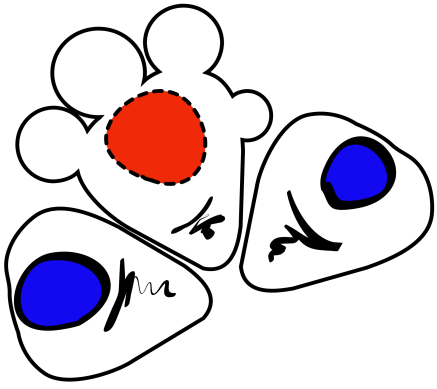
5.1 Visual abstract



1. Histones integrate into acinar cell membranes



2. Histones permeabilise acinar cell membranes, allowing flux of calcium ions and small molecules.



3. Permeabilised cells swell and bleb prior to nuclear permeabilisation and necrosis.

5.2 Background

Data in the previous chapter demonstrate how histones are released into the systemic circulation by cells within the liver, although it is not clear whether this is an active or passive process involving hepatocytes, or resident immune cells. Nevertheless, there is a measurable increase in circulating histones in acute pancreatitis. Furthermore, histones injected into the systemic circulations of mice in the context of cerulein pancreatitis concentrate within the inflamed pancreas to exacerbate pancreatic injury. The mechanism by which histones contribute to cellular necrosis, however, remains unclear.

Histones are positively charged nuclear chaperone proteins that interact with the negatively charged phosphate backbone of DNA to form tightly packed nucleosomes within the nucleus. Modification and breakdown of linker histones within neutrophil nuclei contributes to the massive nuclear volume increase seen immediately prior to NETosis (Papayannopoulos, Metzler et al. 2010; Thomas, Whangbo et al. 2014). Similarly, histones in solution have been shown to interact with negatively charged proteins (Lete, Sot et al. 2014) and phospholipids (Pereira, Marco et al. 1994) at the surface of cells, whereas they do not interact with zwitterionic phospholipids like phophatidylcholine (Pereira, Marco et al. 1994). Addition of a negatively charged phosphate head group increases the strength of histone binding as measured by calorimetry (Lete, Sot et al. 2014). The hypothesis, therefore, must be that histones interact with acinar cell membranes in a charge-dependent mechanism.

In lipid bilayers, histone fragments have been shown to form membrane-spanning pore-like structures (Kleine, Lewis et al. 1997) that increase

conductance to polyvalent cations such as calcium (Kleine, Lewis et al. 1997; Lete, Sot et al. 2014), leading to rapid cellular necrosis. Negatively charged acute phase proteins (such as CRP (Abrams, Zhang et al. 2013)), DNA (Lete, Sot et al. 2014), innate polysaccharides such as heparin (Fuchs, Bhandari et al. 2011) and synthetic macromolecules such as the polypeptide poly-glutamic acid (Gamberucci, Fulceri et al. 1998) all compete with membrane phospholipids and prevent histone integration and toxicity. Bactericidal properties of histone fragments are dependent on their ability to form amphipathic α-helices – potential membrane spanning domains – however no such analyses have been performed on mammalian cells to date (Koo, Kim et al. 2008).

The ability to form trans-membrane pores is of critical interest in acute pancreatitis, due to the possibility of inducing calcium entry into acinar cells. Calcium entry has been shown to be an essential early mechanism in acinar damage (Ward, Petersen et al. 1995; Booth, Mukherjee et al. 2011) and inhibition of calcium entry protects from acinar cell death in several experimental models (Wen, Voronina et al. 2015). Indeed, histones have been observed to increase calcium conductance across the membranes of several other cell types and artificial membranes (Gamberucci, Fulceri et al. 1998). This chapter explores the hypothesis that histones interact with pancreatic acinar cells in a charge-dependent manner, leading to membrane integration, plasmallemma disruption and cell death through unregulated calcium entry.

5.3 Specific methods

5.3.1 Experimental animals

Male CD1 Swiss mice (age 8-10 weeks, weight 30-35 g) were purchased from Charles River UK Ltd (Margate, Kent, UK), housed in a pathogen-free unit with 12 h light-dark cycles and free access to standard lab chow and water. Pancreatic acinar cells were freshly isolated and cell death experiments conducted as set out in the general methods.

5.3.2 Cellular Ca^{2+} measurements

Murine acinar cells were loaded and incubated with fluo-4 (5 μM) or fura-2 (5 μM) Ca^{2+}-sensitive dyes as previously described (Voronina, Collier et al. 2015). Cells were visualised using a Zeiss LSM 510 (Zeiss Microscopy, Cambridge, UK; Ex 488 nm/Em 520 nm) or Till Photonics imaging system (Till Photonics Gmbh, Germany; Ex 340 nm; 360 nm; 380 nm/Em 510 nm) in a perfusion-chamber set-up and challenged with relevant doses of calf-thymus histones. Data for each excitation wavelength as well as the ratio of 340 to 380 excitation were collected.

5.3.3 Reagents

Calf-thymus histones as well as human recombinant histone H2B, H3 and H4 were purchased from Sigma (Sigma-Aldrich, Gilliangham, UK). Fluo4-AM and Fura-2-AM were purchased from ThermoFisher (Thermo, Rockford, USA). Non-specific poly-acetylation of histones was achieved by addition of a molar excess of acetic anhydride, similar to established protocols (Fojo, Reuben et al. 1985). Histones were recovered by solvent evaporation in a fume cabinet and resuspended in PBS prior to use. For some experiments histones were

conjugated with fluorescein isothiocyanate (FITC) and passed through an ion exchange column to remove excess dye according to established procedures (Fattahi, Grailer et al. 2015). FITC-conjugated histones were generated and gifted by Dr Simon Abrams.

5.4 Histones induce pancreatic acinar cell death in a time and dose-dependent manner

To test the hypothesis that histones induce pancreatic acinar cell necrosis at concentrations relevant to acute pancreatitis, freshly isolated murine pancreatic acinar cells were seeded onto a 96 well plate in the presence of 0.1% PI and fluorescent readings taken every minute for 150 minutes. Calf-thymus histones were added to the cell solution after 30 minutes at concentrations selected based on measurements in murine plasma from different experimental models of acute pancreatitis (Ou, Cheng et al. 2015). The lowest histone concentration (50 μg/ml) represents plasma measurements in cerulein pancreatitis, and the highest concentration (200 μg/ml) retro-ductal infusion with tauro-lithocholate. All three concentrations of extracellular histones lead to a rapid increase in PI-related fluorescence, with peak fluorescence measured around one hour after histone application (figure 5-1), before reaching a plateau. When looking at maximal PI-related fluorescence compared to digitonin cell-lysis, 200 μg/ml histones result in near total permeabilization of acinar cells within 60 minutes, and even 50 μg/ml lead to permeabilization of 50% of cells. This finding was confirmed imaging acinar cells one hour after histone treatment with a confocal microscope. There is a dose-dependent increase in cell permeabilization, as well as an unusual pattern of PI-related fluorescence, with not only cell nuclei but also cell membranes fluorescing (figure 5-2).

As calf-thymus histones are a mixture of core and linker histones, the possibility that individual components of this mixture differentially affected the histones ability to induce cell death was investigated. Repeating the cell death experiments with recombinant human histones H2B, H3 and H4, all three core histones were able to induce cellular necrosis (figure 5-1), but histone H3 did so much more effectively than the other core histones at the higher concentration.

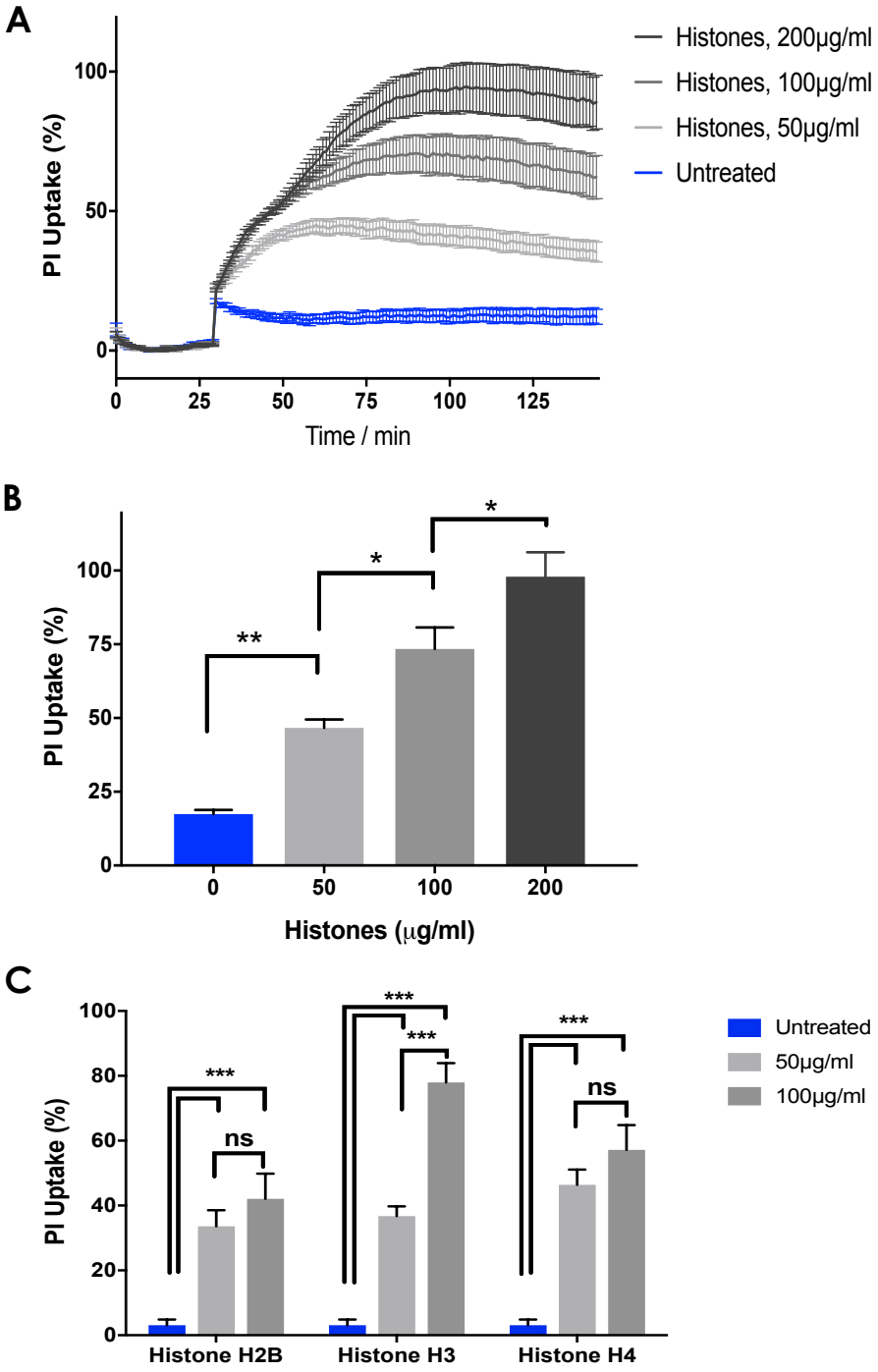


Figure 5-1 A) Propidium iodide-related fluorescence in freshly isolated murine pancreatic acinar cells following administration of different doses of calf-thymus histones over time (mean +/- SEM), as measured on a POLARStar multiwall plate reader. Maximum propidium iodide-related fluorescence in response to increasing doses of **B)** calf-thymus, or **C)** human recombinant histones. All comparisons are by ANOVA. * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Experiments performed in triplicate; $n \geq 4$ experimental repeats.

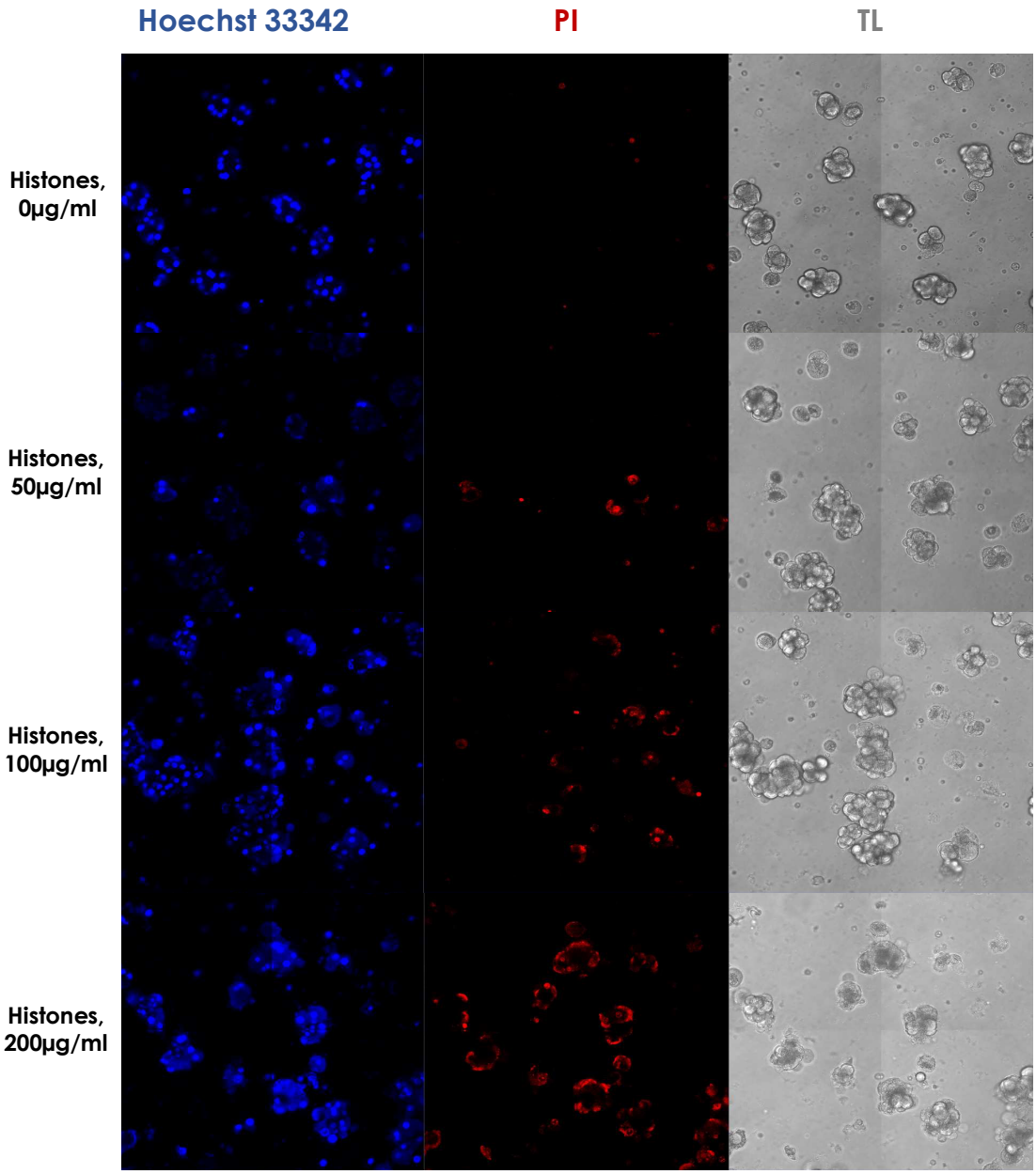


Figure 5-2 Confocal micrograph of freshly isolated murine pancreatic acinar cells 60 minutes following treatment with different concentrations of calf-thymus histones. Fluorescence images represent signals due to Hoechst 3342 (blue) membrane permeable nuclear dye and Propidium Iodide (red), membrane impermeable nuclear dye.

5.5 The histone-acinar cell membrane interaction is mediated by charge

To confirm that histones interact with the cell membrane in a charge-dependent manner before inducing cell death, FITC-labelled histones were added to clusters of freshly isolated murine acinar cells in the presence of the membrane-impermeable nuclear dye PI and imaged with a confocal microscope. Histones concentrate within acinar cell membranes immediately following addition to the solution without any increase in PI signal. Over the next few minutes, some cells swell and become permeable to PI, whereas others in the same cluster remain apparently intact (figure 5-3).

Repeating the cell death experiment with poly-acetylated histones, a modification designed to reduce their overall net-positive charge, reduces their ability to induce membrane permeabilization and cell death. Similarly, co-administration of macromolecules with an overall negative charge protects acinar cells from injury. This protective effect can be overcome by increasing the histone concentration, suggesting a competitive inhibition mechanism (figure 5-3). The protective effect of macromolecules is apparent in inert macromolecules (poly-glutamic acid) as well as the physiological protein albumin.

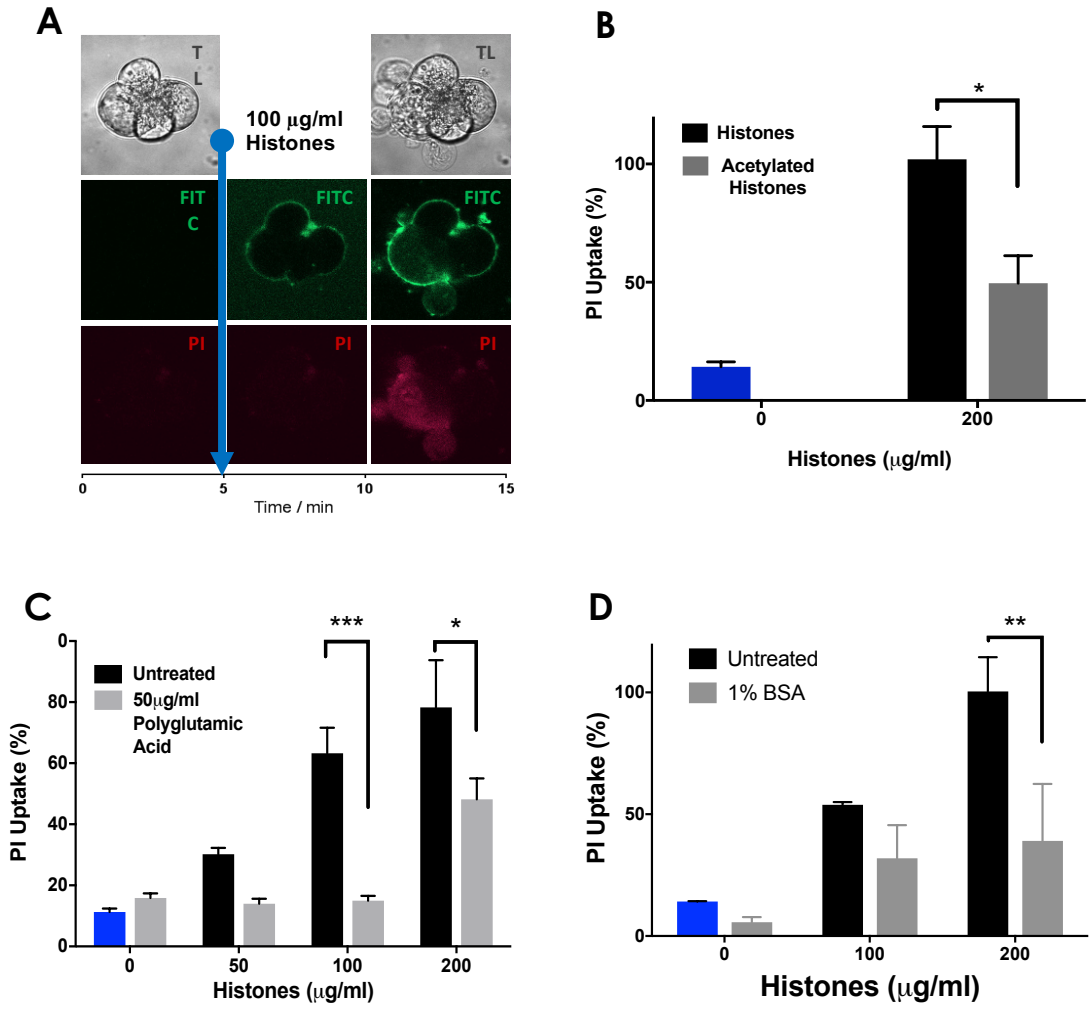


Figure 5-3 A) Representative confocal micrograph of a cluster of murine pancreatic acinar cells over time following treatment with FITC-labelled histones; FITC (green), PI (red). Maximal recorded PI-related fluorescence in freshly isolated murine pancreatic acinar cells after administration of different doses of calf-thymus histones following **B)** poly-acetylation or **C&D)** in the presence of negatively charged macromolecules. All comparisons are by ANOVA. * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Experiments performed in triplicate; $n \geq 4$ experimental repeats.

5.6 Histones increase calcium conductance across acinar cell membranes

As histones have been shown to induce or form non-specific cationic pores in cell membranes and lipid bilayers, the effect of histones on acinar cell calcium conductance was investigated. Using the calcium-sensing fluorescent dye fluo4-AM(Murphy, Criddle et al. 2008), freshly isolated murine pancreatic acinar cells were loaded with dye and imaged using a confocal microscope. An increase in fluorescence signal indicates an increase in intracellular calcium concentration $[Ca^{2+}]_i$. Over 80% of observed cells exhibited multiple typical Ca^{2+} spikes, together with a dramatic decrease in the signal baseline. Cells with the greatest drop in signal baseline exhibited morphological changes consistent with cell death on the transmitted image (figure 5-4).

Fluo4-AM has a molecular weight of around 1 kDa, similar to the 0.67 kDa of PI, raising the possibility that the drop in baseline fluorescence was not secondary to changes in $[Ca^{2+}]_i$, but rather a result of cellular dye loss through membrane leakage. To test this hypothesis, the experiment was repeated using the same setup, except with fura 2-AM as opposed to fluo4-AM. Fura 2-AM similarly has a molecular weight of 1 kDa, but exhibits a ratiometric response to changes in $[Ca^{2+}]$, with the signal at 340 nm excitation increasing in proportion with $[Ca^{2+}]$, and inverse proportion at 380 nm excitation. Moreover, it has a $[Ca^{2+}]$-insensitive isosbestic point at 360 nm, thus allowing differentiation of the $[Ca^{2+}]$ and dye-concentration related effects on signal strength. Figure 5-4 depicts a representative trace of all the typical responses found in acinar cells. Shortly after administration of histones, cells experience true calcium spikes (positive signal changes at 340 nm excitation mirrored by

negative signal changes at 380 nm excitation) with no change in 360 nm signal strength, suggesting constant dye concentration. While some cells will return to baseline signal levels after the initial spike, most exhibit calcium plateaus at increasing [Ca^{2+}] with each calcium spike. All cells then experience signal loss in all three channels, indicative of dye extravasation from the cells.

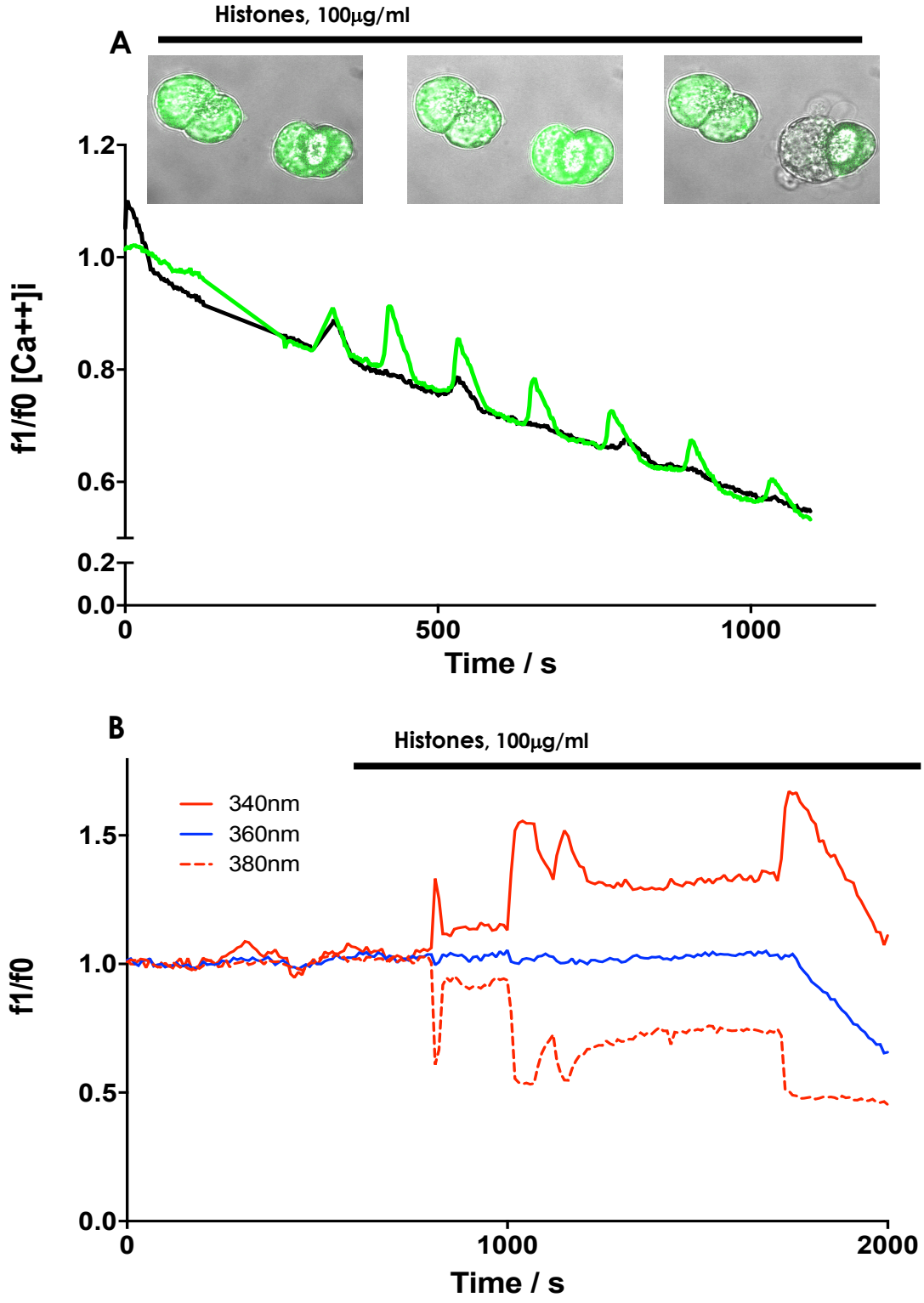


Figure 5-4 Freshly isolated murine pancreatic acinar cell cluster loaded with calcium sensitive dye **A)** fluo4-AM or **B)** the ratiometric calcium-sensitive dye fura-2 AM, following treatment with calf-thymus histones. F1/F0 signal over time.

5.7 Pancreatic acinar cell membrane integrity is disrupted by extracellular histones in a calcium-independent manner

While histones clearly increase calcium conductance in murine pancreatic acinar cells, in a manner that at least initially leads to an appropriate cellular response of calcium potentiation and clearance (Wen, Voronina et al. 2015), the ultimate result is rapid membrane permeabilization to substances of up to 1 kDa. While $[Ca^{2+}]_i$ is critical in the pathogenesis of acinar cell injury in acute pancreatitis (Criddle, Gerasimenko et al. 2007), it is unlikely that a cell could recover from such an insult irrespective of the effects on calcium homeostasis. To test this hypothesis, the fura 2-AM experiments were repeated in buffer containing physiological (1.2 mM) calcium concentrations as in calcium-free buffer. Where many pancreatitis toxins lead to reduced cell death in the absence of calcium (Wen, Voronina et al. 2015), the dye loss induced by histones was as pronounced in the absence as in the presence of extracellular calcium (figure 5-5). Confirming the validity of the experimental setup, the only difference observed in the presence and absence of extracellular calcium was that there were no signal spikes in calcium-free media, consistent with an absence of calcium-induced calcium entry. Furthermore, the degree of dye loss as measured by the mean fura 2 signal loss at 360 nm was proportional to the histone concentration, mirroring the findings of the plate reader cell death experiment.

To provide further support to the hypothesis that acinar cell permeabilization in response to histones is calcium-independent, the cell death experiment was repeated in normal buffer, low calcium buffer with and without calcium chelator and supra-maximal calcium concentrations (figure 5-5D). None of

these experimental conditions had any influence on acinar cell death in addition to histone concentration.

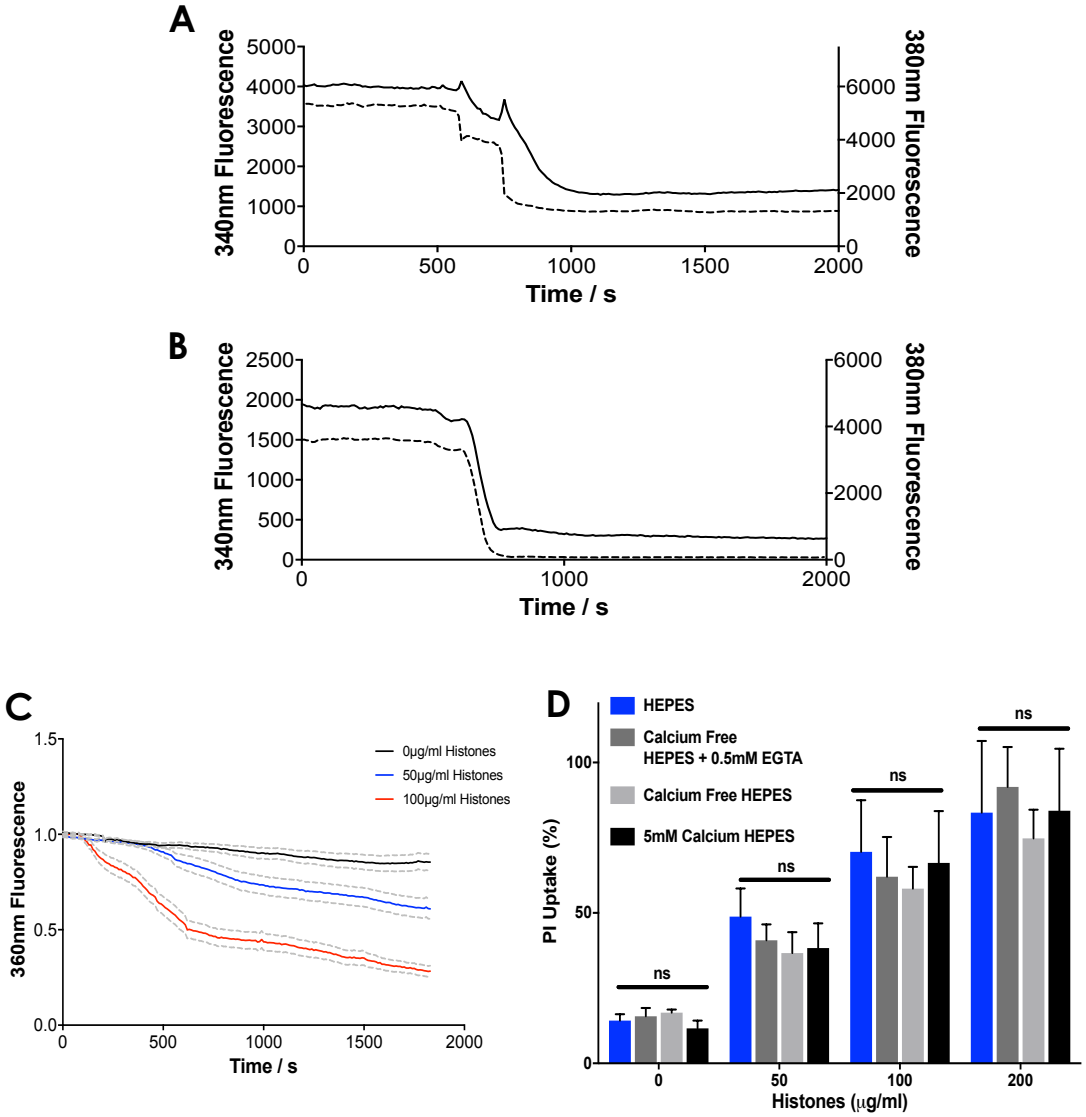


Figure 5-5 Representative trace of fura 2-related signal (340nm solid line, 380nm dashed line) in murine pancreatic acinar cell treated with 100µg/ml calf-thymus histones in the **A)** presence and **B)** absence of extracellular calcium (1.2mM and 0mM). **C)** Mean (+/- SEM) fura 2 – related signal following histone treatment in all observed cells. **D)** Maximal % PI uptake in histone-treated acinar cells in normal, calcium free and calcium excess containing buffers. Comparisons are by ANOVA, observations made in triplicate with n ≥ 4 experimental repeats.

5.8 Discussion

Acute pancreatitis is characterised by high levels of extracellular histones in circulation. The levels tested in the *in vitro* set-up presented here are consistent with those found in experimental models of acute pancreatitis (Ou, Cheng et al. 2015), and also in human acute pancreatitis (Liu, Huang et al. 2017) or acute respiratory distress symdrome (Lv, Wen et al. 2017). Although levels in humans are reported closer to 50 μg/ml, concentrations in excess of 500 μg/ml have been measured in patients with severe disease. When applied directly to isolated pancreatic acinar cells, all of these concentrations can induce acinar cell necrosis.

The data presented here support the hypothesis that positively charged histones interact with negatively charged proteins and/or phospholipids at the surface of cell membranes to facilitate their effects, demonstrated particularly well by reduced toxicity when reducing histone charge. Histones can, thus, be detoxified using negatively charged macromolecules or by reducing the net-positive charge of histones. This leads to the interesting hypothesis that the concentration of histones within the inflamed pancreas (see previous chapter) is facilitated by an exposure to the extracellular matrix and net-negatively charged proteoglycans such as heparin sulfate. It also allows the hypothesis that histones preferentially bind apoptotic cells exposing phosphatidyl sereine residues – the basis of Annexin V staining. Histones have been shown to expose phosphatidyl sereine on the surface of erythrocytes, for example, although it is unclear whether this is as a result of altered flippase activity or through apoptosis pathways (Semeraro, Ammollo et al. 2014).

Histones H3 and H4 are most frequently reported to increase in plasma of patients and experimental animals with acute inflammatory conditions and therapeutic administration of antibodies to these histones improve outcomes in these models (Liu, Ni et al. 2013; Kang, Zhang et al. 2014). Post-translational core histone modification, hyper-citrullination in particular, renders histones H3 and H4 less susceptible to degradation and prevents chromatin packing by linker histones (Wang, Li et al. 2009). It is conceivable that specific histones exhibit greater toxicity, as suggested by the results here, but histones readily oligomerize in solution (Ramakrishnan 1997) and in the presence of a cellular milieu there will always be an ample supply of nuclear proteins, making true differential effects difficult to investigate in this setting. Nevertheless, the finding that of all the recombinant histones tested only H3 exhibited a dose-dependence to its toxicity may suggest that while other subtypes contribute to toxicity, H3 is the necessary limiting factor.

The experiments looking at calcium entry in response to histones demonstrate that histones increase acinar cell permeability to calcium. Furthermore, initially, this is associated with calcium spikes, typically observed in acinar cells as a result of calcium-induced calcium entry (Lur, Sherwood et al. 2011; Wen, Voronina et al. 2015). The amplitude of calcium spikes seen with Fluo-4, however, decrease with each spike, and the drop in baseline is suggestive of dye loss, a suspicion confirmed when looking at the calcium-independent wavelength of fura-2. Critically, removal of extracellular calcium abolishes calcium spikes, but does not protect acinar cells from necrotic cell death, indicating that acinar cell permeabilization is due to the creation of larger

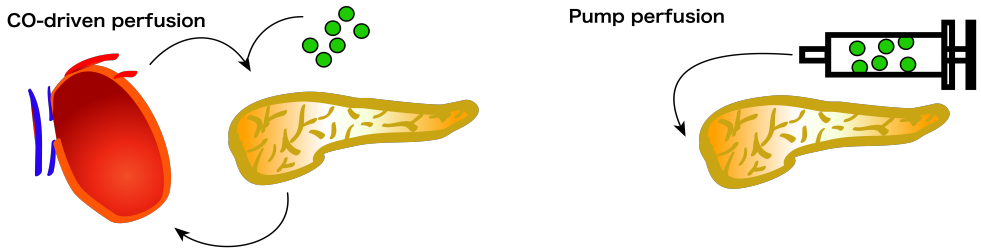
pores that allows flux of ions and larger molecules, at least up to around 1 kDa, the molecular weight of propidium iodide.

5.9 Summary

Extracellular histones disrupt the plasmallemma of acinar cells in a dose and charge-dependent manner. This effect leads to permeabilization of the cells and rapid cell death in a calcium-independent process.

6 Pancreatic Micro-perfusion is Impaired During Acute Pancreatitis

6.1 Visual abstract



1. Development of two novel methods to investigate blood flow in murine acute pancreatitis.

2. Acute pancreatitis leads to patchy perfusion of the murine pancreas.

3. Medium-sized blood vessels take on an irregular appearance / are partially occluded in acute pancreatitis.

6.2 Background

The anatomy of the pancreas, including its blood supply, are very well described. However, there are significant anatomical differences between murine and human pancreata (Dolensek, Rupnik et al. 2015). Whereas in humans the pancreas is a clearly defined organ, fixed anatomically adjacent to the duodenum by its peritoneal covering, in mice and other quadruped mammals it is distributed in three major parts throughout the proximal small bowel mesentery (Liu, Xue et al. 2010). The arterial supply of the gland is highly variable in humans (Macchi, Picardi et al. 2017), with blood being supplied by three major vessels: the common hepatic artery, the splenic artery and the superior mesenteric artery (Bertelli, Di Gregorio et al. 1995; Bertelli, Di Gregorio et al. 1996; Bertelli, Di Gregorio et al. 1996; Bertelli, Di Gregorio et al. 1997; Bertelli, Di Gregorio et al. 1998). The blood supply to the head and body/tail of the pancreas is functionally distinct allowing surgical resection of part of the gland. There are no detailed studies of murine pancreatic blood supply, although it is macroscopically considered similar to that of the human pancreas (Dolensek, Rupnik et al. 2015). Microscopically, however, there are important differences between the murine and human pancreas. In humans, pancreatic islets are located within acinar lobules, and blood flows from the major arterioles to the islets in the first instance, before supplying the acinar cells via an insulo-acinar portal circulation (Murakami, Hitomi et al. 1997). In mice, islets are generally located between acinar lobules and blood from islets drains directly into the portal venous system (Murakami, Fujita et al. 1993). Critically, all venous blood from the pancreas has to pass via the portal vein through the liver before it enters the systemic circulation (see Chapter 3).

At rest, the pancreas receives approximately 1% of total cardiac output, and blood flow has been measured using a wide variety of different techniques ranging from plethysmography to microsphere studies (Lewis, Reber et al. 1998), and more recently intra vital microscopy to evaluate capillary perfusion (Schmidt, Ebeling et al. 2002; Ryschich, Kerkadze et al. 2009). Early studies of pancreatic blood flow made use of large mammals (frequently dogs), due to the technical difficulties in attaching the sub-millimeter blood vessels found in rats and mice to recording instruments (Mandelbaum and Morgan 1969; Goodhead, Himal et al. 1970; Studley, Mathie et al. 1987) and relative familiarity with the canine pancreas following the work on insulin (Banting, Best et al. 1922). Recent studies of murine pancreatic blood flow concentrate on microcirculatory disorders (Chen, Sunamura et al. 2001), but as a technique neither intra-vital microscopy nor microbeads can provide useful information with regard to the medium vessel-level perfusion of the murine pancreas.

Ischaemia-reperfusion injury is a concept with its origin in cardiac research, whereby reperfusion of cardiac tissue after a period of ischaemia induces further tissue injury secondary to radical oxygen species (ROS) generation (Donato, Evelson et al. 2017). Mitochondrial calcium concentration, the ability of mitochondria to generate an electrochemical gradient and dissipating that gradient through opening of a mitochondrial permeability transition pore (MPTP) are all factors that contribute to myocardial injury following ischaemia (Andrienko, Pasdois et al. 2017). Similarly, mitochondrial calcium, opening of the MPTP and mitochondrial ROS generation are all critical factors in acinar cell injury following non-primary ischaemic insults to the pancreas (Criddle, Gerasimenko et al. 2007; Mukherjee, Criddle et al. 2008; Booth, Murphy et al.

2011). One of the cardinal features of myocardial ischaemia-reperfusion injury, is that repeated episodes of sub-clinical ischaemia protect from a major future ischaemic event – so called ischaemic preconditioning (Donato, Evelson et al. 2017). Ischaemic preconditioning has been shown to reduce the severity of ischaemia/reperfusion-induced acute pancreatitis in rats, but more interestingly short periods of pancreatic ischaemia (clamping of the coeliac artery) has also been shown to be effective in reducing pancreatic injury and inflammation following cerulein-induced acute pancreatitis (Warzecha, Dembinski et al. 2005; Warzecha, Dembinski et al. 2007). This suggests either that the protective mechanisms involved in ischaemic preconditioning protect from a multitude of inflammatory conditions, or that ischaemia is a hallmark of acute pancreatitis, even if induced by cerulein.

Abnormalities of blood flow, in particular capillary occlusion manifesting in a reduction in capillary density, have been observed during acute pancreatitis in a number of different experimental models (Nuutinen, Kivisaari et al. 1986; Plusczyk, Westermann et al. 1997), and similarly reduced pancreatic blood flow has been observed in patients with acute pancreatitis using cross sectional imaging (Delrue, Blanckaert et al. 2012; Watanabe, Tsuji et al. 2013; Yadav, Sharma et al. 2015). The aim of the following set of experiments was to develop experimental techniques to assess pancreatic blood flow in the same 12 cerulein injection murine model used repeatedly thus far and to assess uniformity of blood flow within the inflamed pancreas at a whole-organ level.

6.3 Specific methods

6.3.1 In vivo/in situ perfusion

Two different perfusion methodologies were trialled: cardiac output-driven self-perfusion, and *ex vivo* pump perfusion. The principles of microsphere perfusion were similar to previously published methods (Deveci and Egginton 1999) and following the principles set out by the Fluorescent Microsphere Resource Centre (University of Washington, Seattle, WA, USA; fmrc.pulmcc.washington.edu – accessed April 2015). For imaging in acute pancreatitis, perfusion was commenced one hour after the last cerulein injection in a 12-hourly injection model of cerulein acute pancreatitis (see common methods).

For determination of whole-organ blood flow, CD1 Swiss-type mice had their left carotid arteries and left femoral arteries cannulated under terminal anaesthesia using a dissecting mircoscope. Carotid artery cannulae were advanced just beyond the thoracic inlet and secured using a bulldog clip. Arterial cannulae were locked with 200 IU unfractionated heparin. 140,000 microspheres were injected via the carotid artery cannula in a slow bolus over 60 seconds in 500 µl of PBS using a fixed-rate infusion pump, while simultaneously withdrawing blood from the femoral artery cannula. Animals were culled by anaesthetic overdose following the sampling process, and the pancreas, kidneys and lung were extracted. 36,000 Blue-green control microspheres were added to tissue and blood samples, which were then digested for 48 h in 2.3 M ethanolic KOH with 0.5% Tween 80 at 50 °C, vortexing at 24 and 48 h and sedimented at 2000 xG for 20 minutes. The sediment was then rinsed and re-centrifuged twice with 1% Triton X-100 prior to dissolution of

microspheres in 2-ethoxyethyl acetate in a fume cabinet. Samples were centrifuged again after 24 hours at 2000 xG for 20 minutes and fluorescence in the supernatant measured using a BMG POLARstar Omega Microplate Reader (Imgen Technologies, New York, USA). Microspheres are resistant to KOH digestion, but are readily dissolved by 2-ethoxyethyl acetate, allowing retrieval of microspheres from tissue with this digestion/sedimentation method, prior to releasing the pure fluochrome ready for measurement. Addition of control microspheres at the beginning of the digestion process allows for an estimate (and correction) of microspheres during the repeated wash/sedimentation steps. Fluorescence was. To count as a successful experiment, fluorescence in lung tissue should be low (indicating limited re-circulation of microspheres) and difference in fluorescence between both kidneys should be less than 20% (indicated adequate mixing upon carotid injection). Fluorescence in the known volume of blood sampled over 60 seconds from the femoral artery was used to calculate pancreatic blood flow as a ratio of fluorescence.

For the imaging of regional pancreatic perfusion, CD-1 Swiss mice (Charles River, UK) were culled by overdose of anaesthesia, and their abdominal aorta quickly cannulated using a dissecting microscope on a heated table (Figure 6-1). Upon cannulation, a bulldog clamp was applied across the aorta immediately proximal to the left renal artery and a second clamp to the thoracic aorta just above the diaphragm. The aorta was the perfused with 0.9% Saline at a rate of 1 ml / min for 2 minutes, before 10-minute perfusion with a solution of 3% agarose (ultra-low gelling point) containing either 10% TRITC-dextran, 1 µm-diameter fluorescent microspheres, Gd(III)-based MRI

contrast agent (Magnavist) or FeO. This perfusion method resulted in near isolated pancreatic perfusion, as detailed in figure 6-2. Retrograde perfusion via the hepatic portal vein was also trialled (figure 6-3) as this is technically much more straight-forward, however this resulted in global perfusion of the small bowel mesentery. Immediately following cessation of perfusion, animal corpses were placed in a freezer at -4 °C to ensure gelling of intra-vascular agarose solution, similar to previously published methods (Dutly, Kugathasan et al. 2006). Whole pancreata, including proximal duodenum and spleen, were then extracted and imaged using either an IVIS Spectrum preclinical imaging system (Perkin Elmer, Waltham, MA, USA), or immobilised in a 10% agarose gel within a 7 ml Bijou tube, prior to imaging with a 9.4 T Bruker Biospec 94/20 USR MRI machine (Bruker UK Ltd, Coventry, UK). Fluorescent images were collected using IVIS fluorescence unmixing settings to separate dye-specific and tissue background fluorescence. Intensity was measured using Image J 1.51s for Mac (ImageJ 1.51s for Mac, NIH, USA).

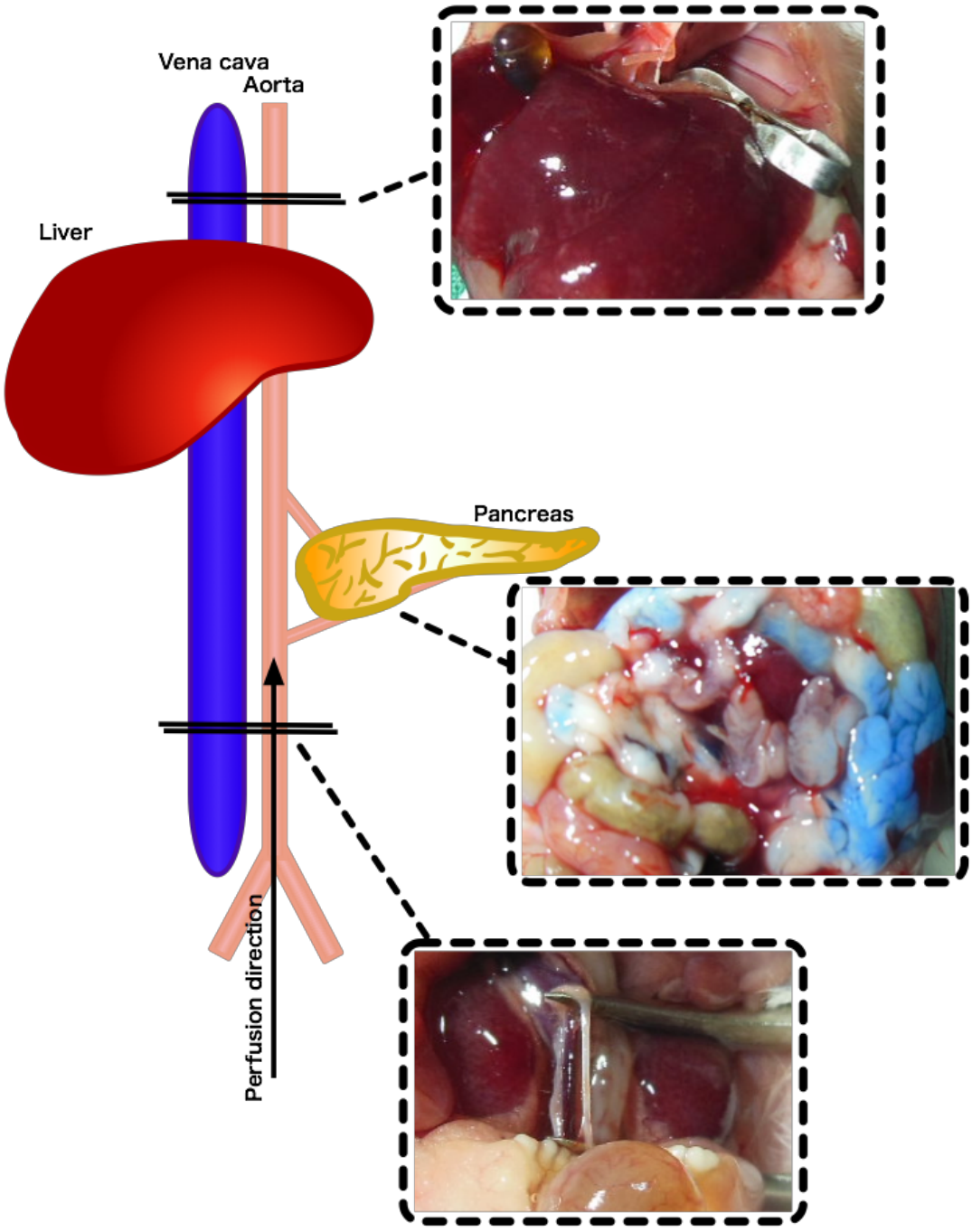


Figure 6-1 Arterial perfusion set-up. Diagram of liver, pancreas, aorta, and vena cava, detailing the set-up for arterial pancreatic perfusion. The black arrow indicates placement of the perfusion cannula. The black parallel lines indicate Bulldog clamps. Picture insets (top to bottom): Bulldog clamp on supra-hepatic vena cava; *in situ* pancreas following perfusion with Methylene Blue; abdominal aorta (lefted by forcepts) next to abdominal vena cava.

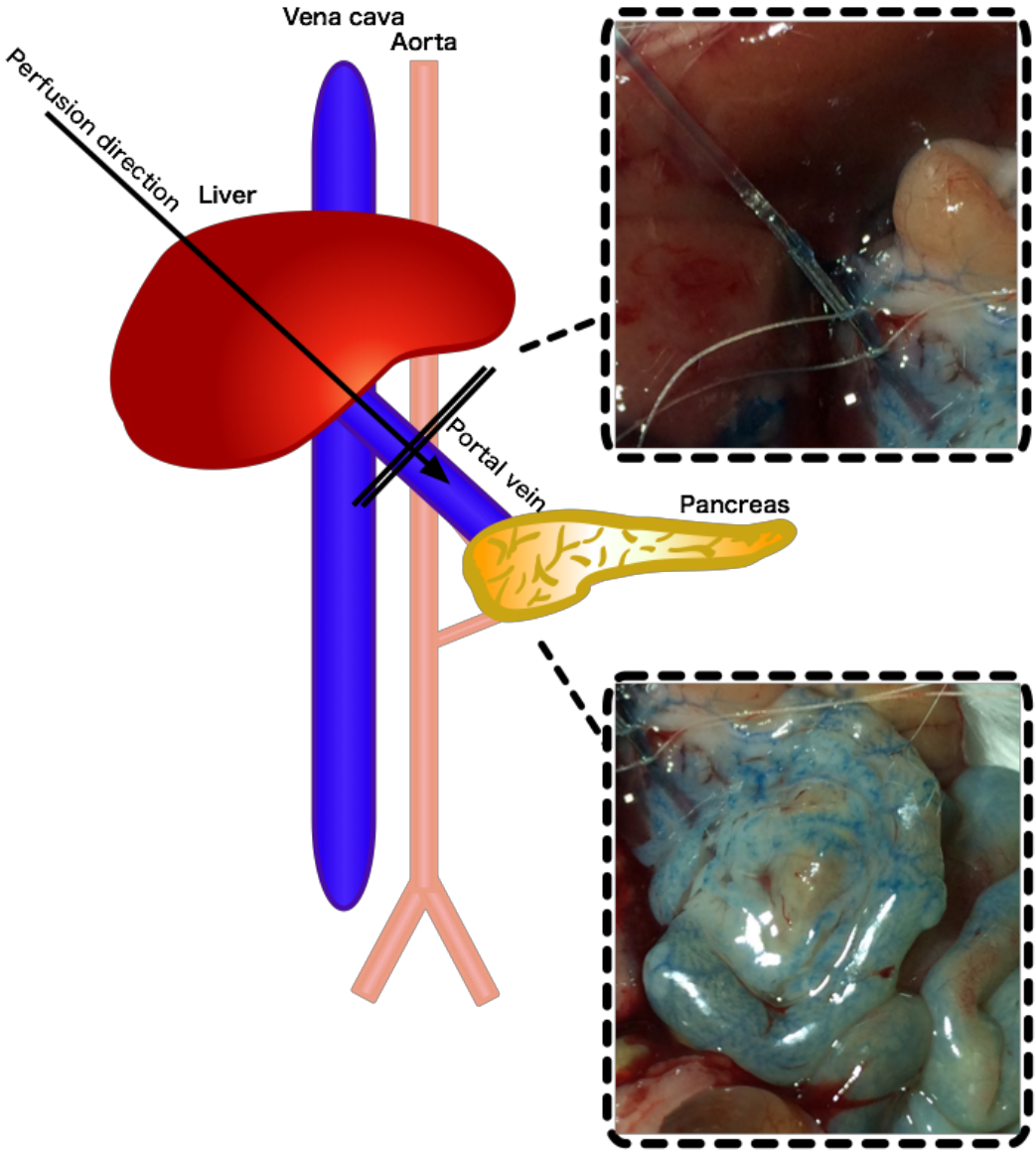


Figure 6-2 Portal venous perfusion set-up. Diagram of liver, pancreas, aorta, portal vein and vena cava detailing the set-up for portal venous pancreatic perfusion. The black arrow indicates placement of the perfusion cannula. The black parallel lines indicate occlusive sutures. Picture insets (top to bottom): perfusion cannula sutured in place within portal vein; *in situ* pancreas and proximal small bowel following perfusion with Methylene Blue.

6.3.2 Reagents

FluoSpheres™ fluorescent microparticles were purchased from ThermoFisher (Life Technologies LTD, Paisley, UK) in 1 and 10 μM diameters, conjugated to yellow/green (505/515), red (580/605) and blue/green (430/465) fluorescent dyes. PBS, agarose (ultra-low gelling point) and TRITC-dextran (average molecular weight 40 kDa) were purchased from Sigma (Sigma-Aldrich Company Ltd, Dorset, UK). Plastic tubing (external diameter 0.8 mm) for vascular cannulation, Gd(III)-based MRI contrast agent (Magnavist) and FeO nanoparticles were gifted by the Centre for Preclinical imaging at the University of Liverpool, UK.

6.4 Total pancreatic blood flow is reduced in experimental acute pancreatitis

Using cardiac output-driven self-perfusion with fluorescent microspheres, pancreatic blood flow could be estimated at around 1.2 ml/min/g tissue. This dropped to below 0.4 ml/min/g tissue in necrotising acute pancreatitis secondary to 12 hourly injections of cerulein (figure 6-4). Mixing of microspheres was deemed adequate in around 75% of experiments (a difference in fluorescence between left and right kidneys or <= 20%), but concurrent femoral artery sampling was only possible in around 50%, due to thrombosis of the first arterial cannula (despite heparinisation) in the time it took to cannulate the second artery. Given that standard poly-ethelene tubing was used in these experiments, it is likely that the more expensive heparin/EDTA impregnated vascular cannulae would result in more reliable experiments.

Where differences between fluorescence measured in the left and right kidney exceeded 20%, pancreatic fluorescence measurements were excluded from the blood flow analysis. It is notable that in all cases, fluorescence in the right kidney exceeded that of the left, suggesting a mechanism other than abnormal mixing, which would be expected to produce random differences in microsphere distribution.

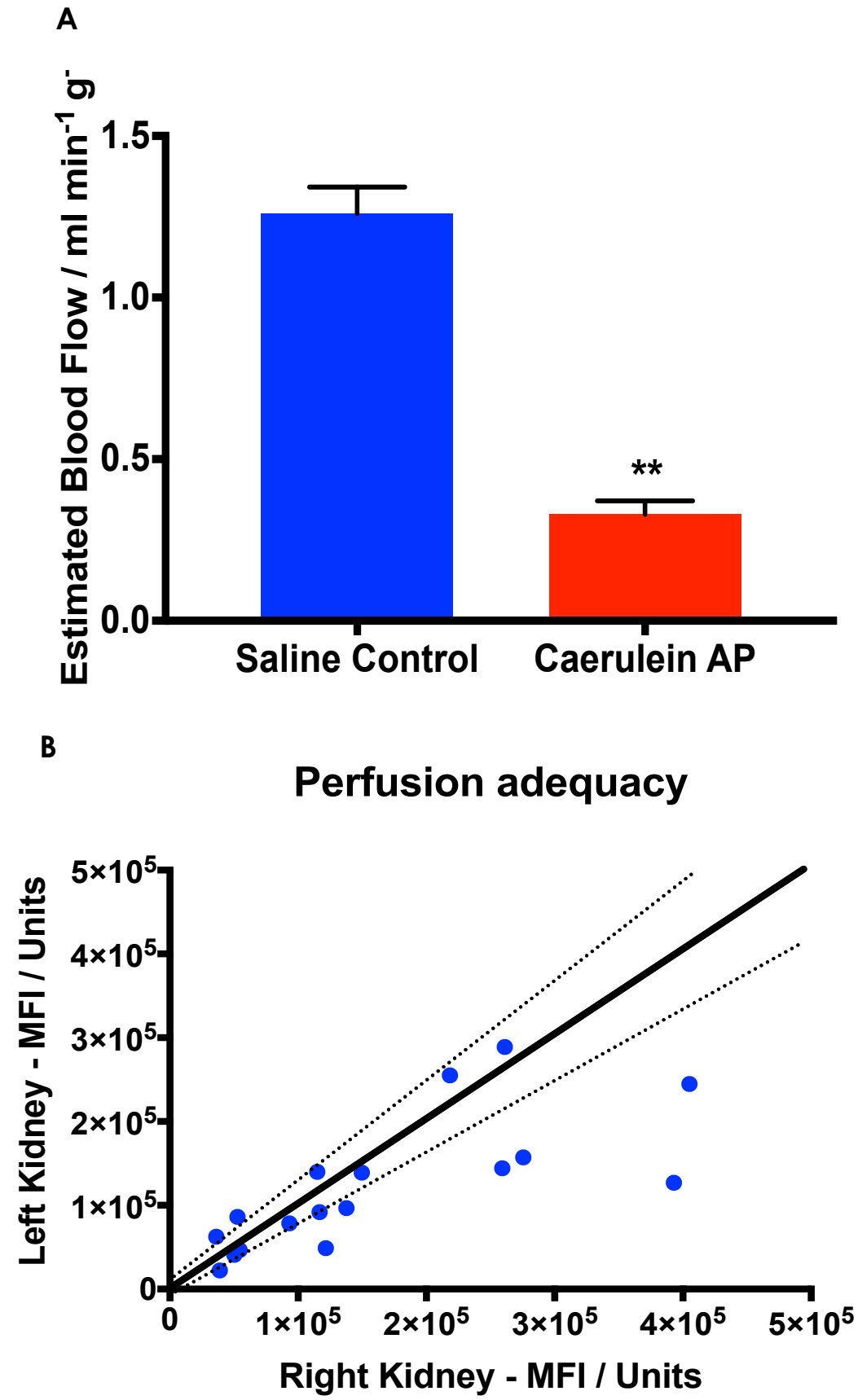


Figure 6-3 A) Murine pancreatic blood flow estimated from pancreatic fluorescence following injection of 15µm fluorescenct microspheres via the carotid artery of CD1 Swiss mice under terminal anaesthesia. and concomitant fluorescence measurement of a known volume of femoral arterial blood collected over 60 seconds. **B)** Comparison of fluorescence in left and right kidneys as indication of even mixing of fluorescent particles. Solid oblique line indicates equivalent fluorescence; dotted line indicates 20% deviation from equivalence. Comparisons were made by Student's t-test, with * denoting $p<0.05$ and ** $p<0.01$; $n \geq 12$.

6.5 Cerulein-induced severe pancreatitis results in patchy organ perfusion and necrosis

Following *in situ* arterial perfusion of mouse pancreata with 1 µm diameter fluorescent microspheres, duodenum, pancreas and spleen of experimental animals were resected *en bloc* and embedded in Bijou tubes within a 10% agarose gel. Imaging these tubes in an IVIS Spectrum epifluorescence chamber revealed whole-organ distribution of fluorescent particles. Figure 6-4 demonstrates that overall pancreatic fluorescence intensity is lower in cerulein acute pancreatits compared to saline controls. Moreover, pancreata of AP animals exhibit patches of non-fluorescence (i.e.: hypo-perfusion) not seen in that of saline controls.

Histology reveals extensive necrosis in terminal lobules with intravascular thrombosis in lobular feeding vessels with near normal adjacent pancreatic tissue. This pattern of near total necrosis is only seen in association with terminal arterioles at the peripheries of the gland. Centrally, the more typical diffuse pattern of necrosis is observed.

MRI imaging of the same pancreata reveal details of the vasculature down to a size of 0.1 mm. Similar to fluorescence and histological data, areas of near-normal appearance can be seen in cerulein AP pancreata, with patches of oedema (seen as bright white on Gd(III)-enhanced, T1-weighted images) associated with an obliteration of the normal microvascular pattern. Using FeO nanoparticles as contrast agent, major arteries traversing the pancreas can be seen as clearly patent in cerulein AP pancreata, with clear irregularities in some major vessels and patches of absent perfusion (figure 6-5).

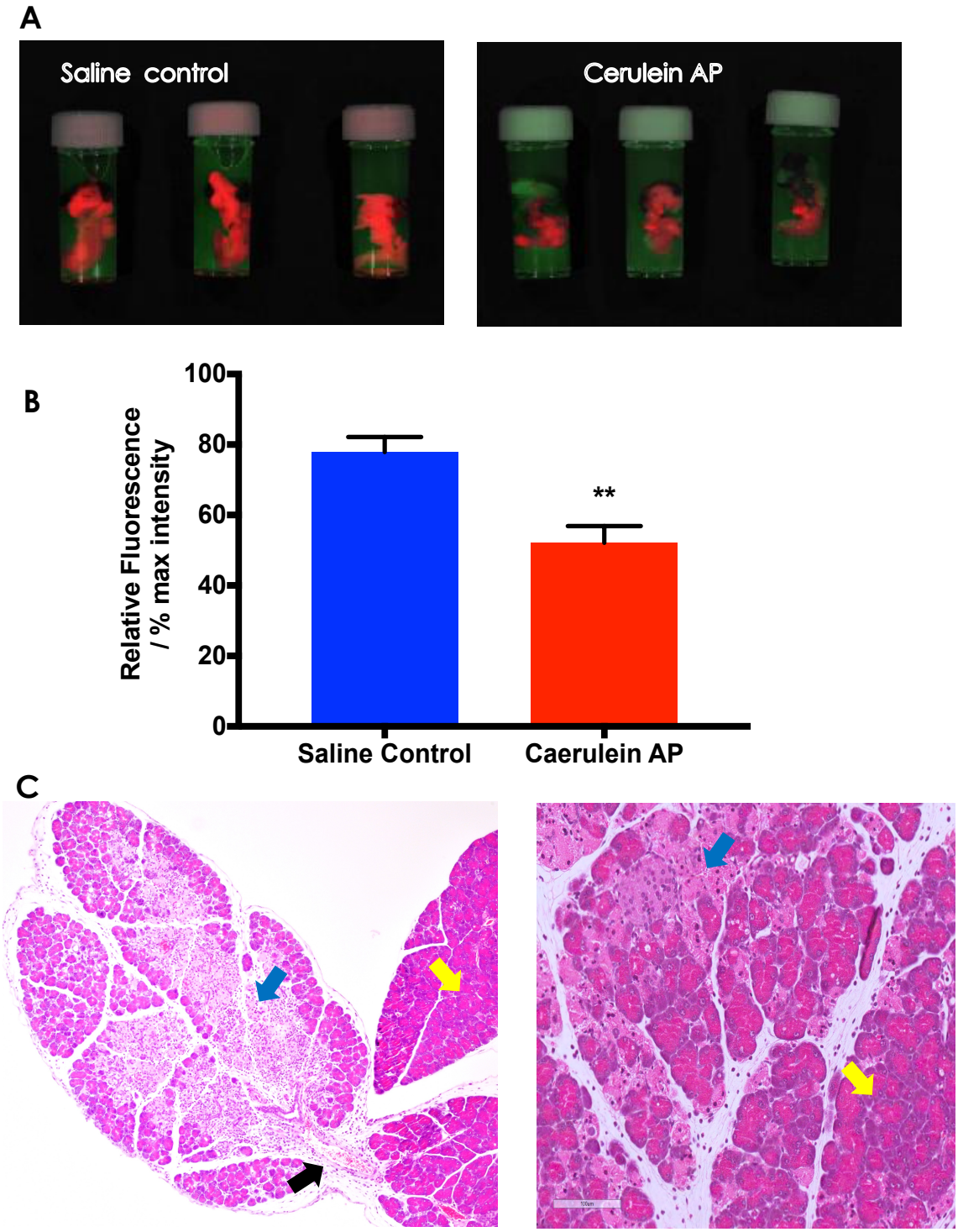


Figure 6-4 Fluorescence measurement of mouse pancreata perfused with fluorescent microspheres in cerulein-pancreatitis or saline controls. **A)** epifluorescence chamber image of microsphere-perfused murine pancreata suspended in agarose gel. **B)** whole-organ fluorescence following homogenisation and dye extraction from perfused pancreata. **C)** H&E-stained micrographs of pancreata from two distinct cerulein-treated mice at 100x and 400x magnification, demonstrating areas of significant necrosis (blue arrow) adjacent to near-normal tissue (yellow arrow) as well as thrombosis of a peripheral arteriole causing near total lobular necrosis (black arrow). Comparisons are by Student's t-test, with * denoting $p<0.05$ and ** $p<0.01$; $n \geq 8$.

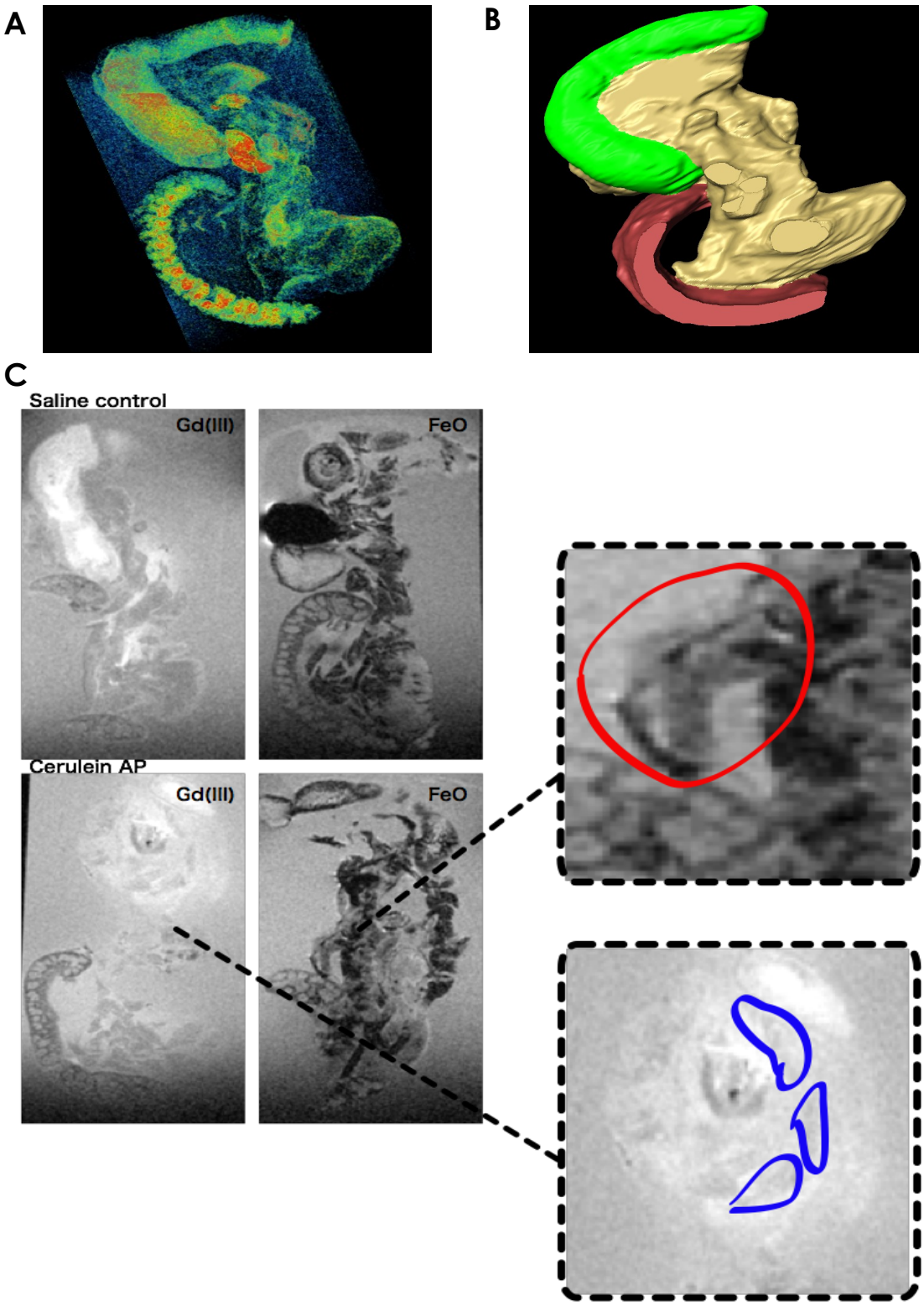


Figure 6-5 MRI images of pancreata from mice treated with 12x hourly injections of 50µg/kg caerulen or saline controls. **A&B)** 3D reconstruction and volumetry of specimen, where yellow/red indicates areas of high perfusion in **A,** and **B** is colour-coded by organ (green = duodenum, yellow = pancreas and red = spleen). **C)** T1-weighted images with Gd(III)-based contrast medium and T2-weighted images with FeO nanoparticles as contrast agent. Insets demonstrate vascular discontinuity and irregularity (red) and areas of hypoperfusion (blue).

6.6 Discussion

Perfusion defects are a hallmark of acute pancreatitis, including hyperstimulation AP (Hackert, Pfeil et al. 2007). Using a number of different techniques, whole-organ perfusion was evaluated qualitatively and quantitatively in the murine pancreas using a severe cerulein hyperstimulation model of AP. This demonstrated a dramatic reduction of pancreatic blood flow immediately after induction of AP, as well as irregularities of medium-level vessels and patches of relative hypo-perfusion adjacent to near normal pancreatic tissue. The reperfusion that must follow this period of relative ischaemia was not investigated here, but presumably will contribute to the eventual area of tissue damage and necrosis, although the complex blood supply to the pancreas, through its multitude of inflow vessels with numerous collateral supply vessels, mean that confluent necrosis was only every observed in peripheral lobules supplied by single vessels, a feature which may limit added reperfusion injury by reducing the likelihood of complete ischaemia.

Areas of necrosis always correlate with areas of extensive inflammatory infiltrate. The assumption had always been that DAMPs and chemokines released by injured and destroyed acinar cells recruit inflammatory cells, however it is possible that recruited inflammatory cells themselves contribute to intra-vascular thrombosis, thereby contributing to further ischaemia. Similar phenomena have been reported in AP (Ryschich, Kerkadze et al. 2009), in experimental venous thrombosis (Brill, Fuchs et al. 2012; Martinod, Demers et al. 2013) and arterial injury (Fuchs, Brill et al. 2010; Michels, Albanez et al. 2016), where histones or NETs interact with adjacent endothelium, thrombocytes and

neutrophils to induce and establish thrombus formation in inflamed tissue in otherwise healthy vasculature. Having demonstrated the presence and significance of extracellular histones in AP in previous chapters, one can postulate that the inflammatory infiltrate contributes to local ischaemia and, thereby, exacerbates tissue injury. In sepsis models it has been shown that such an effect can limit the spread of micro-organisms (McDonald, Urrutia et al. 2012) and Group A Streptococcus has been found to express DNAse, allowing it to degrade NETs and produce rapidly spreading cellulitis and necrotizing fasciitis (Buchanan, Simpson et al. 2006). It is conceivable that a similar mechanism limits the injury caused by activated pancreatic proteases, explaining the lack of positive feedback and total necrosis seen in most patients with acute pancreatitis. This hypothesis would, however, also suggest that there is a protective function of immune-thrombosis in AP.

Several ischaemic clinical conditions have been found to have a 'golden window' of therapy, where earlier re-perfusion reduces tissue injury and long-term functional outcome. This is the case in ischaemic stroke (Osborn, LaMonte et al. 1999) and myocardial infarction (Heestermans, van 't Hof et al. 2010), and the concept of a 'golden hour' of therapy has been more widely adopted in sepsis (Kumar, Roberts et al. 2006; Nugent and Coopersmith 2017). The only effective therapy in the management of acute pancreatitis, administration of intravenous fluid replacement, has similarly been shown, in a large international multicentre trial, to be more effective if given early (Singh, Gardner et al. 2017). Experimentally, however, limiting immune-thrombosis prior to induction of acute pancreatitis has had mixed results in AP, sometimes exacerbating tissue injury (Ryschich, Kerkadze et al. 2009;

Warzecha, Dembinski et al. 2012). This leads to the possibility that there is indeed a 'golden' therapeutic window, but that unlike in other, purely ischaemic pathologies this window does not start with the onset of the disease.

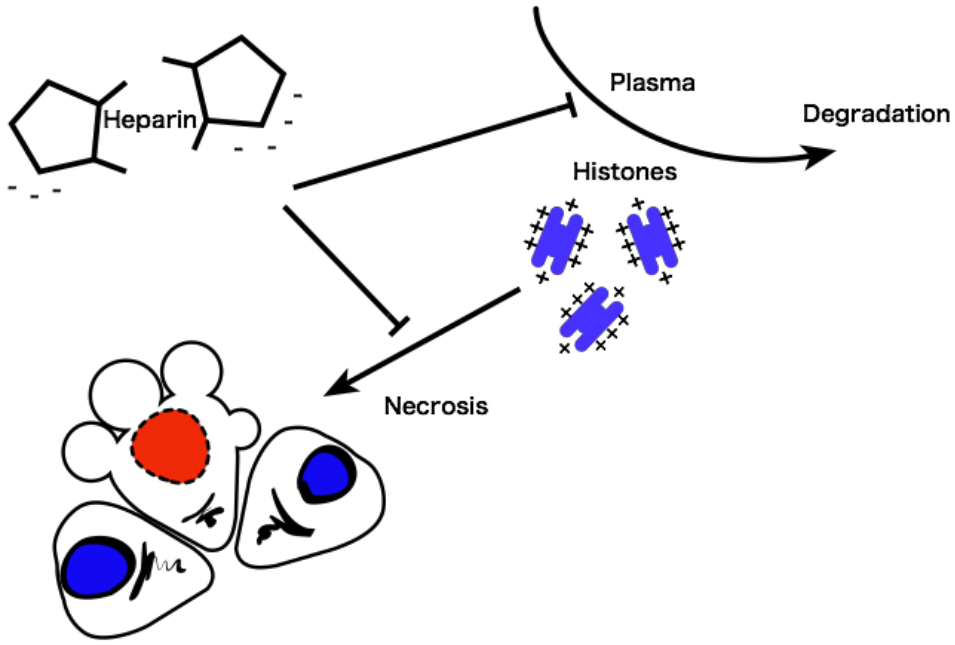
The perfusion techniques developed and described in this chapter are effective in measuring total pancreas blood flow in the mouse both at baseline and in acute pancreatitis, adding a further element of detail to the investigation of the disease and its therapies. The imaging of larger and medium sized vessels is of interest and has added to the understanding of the pathogenesis of the cerulein model, however it is likely too laborious, expensive and of limited value due to the resolution limitations of the MRI machine to be adopted more widely. Nevertheless, further development of this technique could allow *in vivo* imaging of pancreatic perfusion in mice, thereby introducing a further clinically relevant measure of pancreatic injury in pancreatitis with the potential to significantly reduce the number of experimental animals required for therapeutic studies.

6.7 Summary

Pancreatic blood flow is dramatically reduced in cerulein-induced acute pancreatitis and is associated with irregularities in major vessels as well as patches of significant hypo-perfusion throughout the gland.

7 Histones and NETosis as Therapeutic Target in Acute Pancreatitis

7.1 Visual abstract



Acute pancreatitis + Heparins (UFH/ODS/LMWH)
= no therapeutic benefit.

Acute pancreatitis + PAD4 Inhibitor (GSK484/Cl-amidine)
= reduction in tissue injury and inflammation.

7.2 Background

Perfusion defects or other detrimental effects on pancreatic microvasculature have been widely reported in experimental animals as well as human patients. In particular, constriction at the inter-lobular arteriolar sphincter (Zhou, Chen et al. 2002) and leukocyte adhesion at post-capillary venules (Schmidt, Ebeling et al. 2002; Hackert, Pfeil et al. 2007; Ryschich, Kerkadze et al. 2009) together result in a reduction in overall functional capillary density and reduced blood flow through capillary beds. Pancreatic perfusion defects can also be observed in patients, where they are either temporary or permanent, resulting in pancreatic necrosis (Tsuji, Yamamoto et al. 2007; Delrue, Blanckaert et al. 2012; Yadav, Sharma et al. 2015). Hepatic arterial perfusion has also been reported to increase in acute pancreatitis, without significant difference in portal blood flow, resulting in an overall increase in total hepatic blood flow seen in patients (Koyasu, Isoda et al. 2012) and experimental animals (Tutcu, Serter et al. 2010), possibly as a result of increased intra-pancreatic vascular resistance.

Critically, defects in pancreatic perfusion can be modified and improved. Vasoactive substances such as endothelin-1 or platelet activating factor promote microcirculatory dysfunction, and their inhibition can normalise blood flow and reduce tissue necrosis (Foitzik, Hotz et al. 1999). Protease inhibition also improves blood flow (Keck, Friebe et al. 2005), although the mechanism could be inhibition of immune-thrombosis (Pfeiler, Massberg et al. 2014). Anticoagulants such as heparin have also been used to improve pancreatic blood flow and reduce necrosis with some success, both in continuous regional arterial perfusion application (Ke, Ni et al. 2014) and as

pre-treatment (Ceranowicz, Dembinski et al. 2008; Koksoy, Yankol et al. 2013), although heparin has also been observed to abolish the beneficial effects of ischaemic preconditioning (Warzecha, Dembinski et al. 2012), suggesting that an initial period of transient ischaemia may be beneficial in acute pancreatitis.

Heparin is a polysulfated carbohydrate present in mammalian gut (Li and Corey 2013) and its anti-coagulant activity depends on its interaction with antithrombin III, a charge mediated phenomenon dependent on multiple sulphate groups (Rao, Argyle et al. 2010). Heparins have well recognised anti-inflammatory properties (Young 2008) and their high net-negative charge density make them likely competitive inhibitors of histone-induced toxicity. In light of the impairment in pancreatic microcirculation as well as histone toxicity described in previous chapters, the role of different heparin fractions in the treatment of cerulein-induced acute pancreatitis was evaluated.

7.3 Specific methods

7.3.1 Experimental models

The 12 injection cerulein model was used throughout the experiments here described using the methods detailed in the general method section. All pre-treatments were given one hour prior to the first cerulein injection and all treatments were administered 3 hours after induction of acute pancreatitis with the 4th cerulein injection.

7.3.2 Reagents

Unfractionated heparin (UFH) was purchased from Wockhardt (Wockhardt UK Limited, Wrexham, UK), Tinzaparin sodium (LMWH) from Sigma (Sigma-Aldrich

Company Ltd, Dorset, UK). O-desulfated heparin (ODS) – a heparin with very low anti-coagulant properties – was gifted from Cantex (Cantex Pharmaceuticals, Weston, FL, USA). Cl-amidine was from Calbiochem (Merck-Millipore UK Ltd, Watford, UK) and GSK484 was from Cayman Chemicals (Cambridge Bioscience Ltd, Cambridge, UK).

7.4 Heparins protect pancreatic acinar cells from histone-induced injury

Using a similar experimental set-up as in chapter 5, freshly isolated murine pancreatic acinar cells were seeded onto 96-well plates and Propidium Iodide-related fluorescence measured after 60-minute exposure to increasing concentrations of calf-thymus histones. In the absence of heparin, histones produced the familiar dose-dependent increase in PI-related fluorescence. Unfractionated heparin (UFH) was able to eliminate histone toxicity without exhibiting toxicity of its own. 2-0, 3-0 desulfated (ODS) heparin, a modified heparin with very low anticoagulant effect in vivo, was similarly able to reduce histone-induced toxicity, although did exhibit acinar cell toxicity of its own in the absence of histones (Figure 7-1).

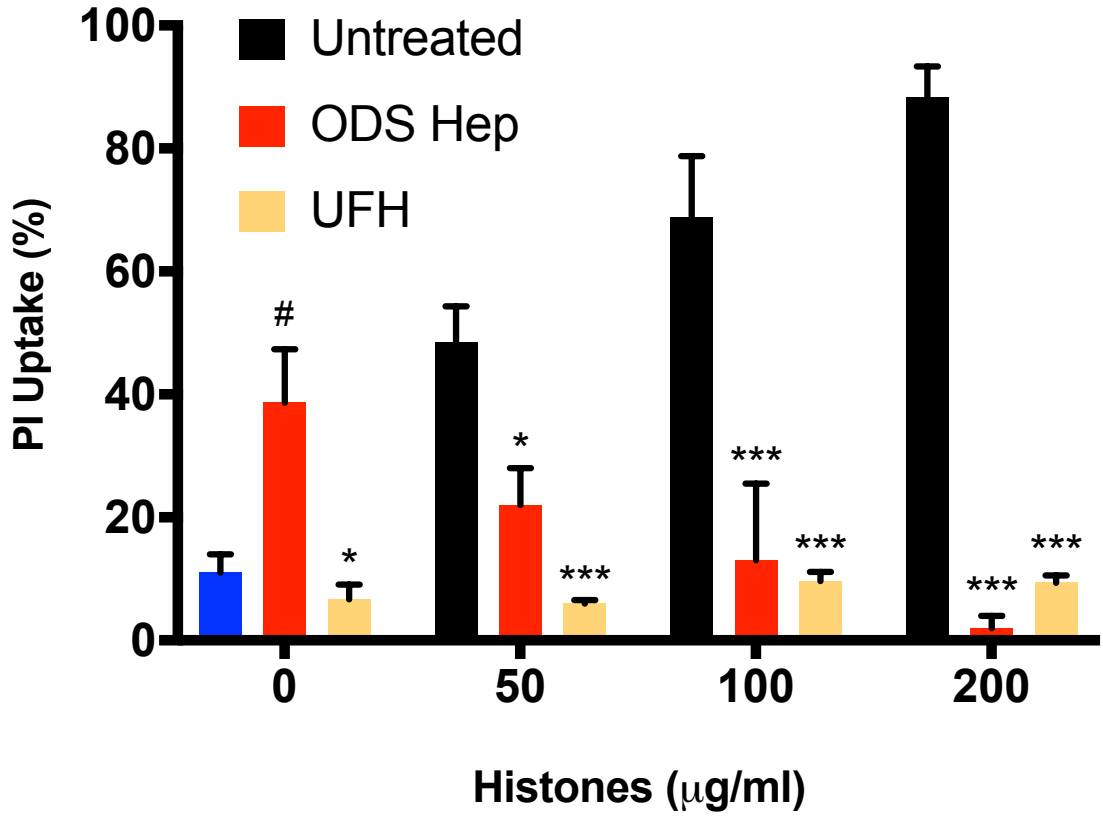


Figure 7-1 Maximal recorded PI uptake in freshly isolated murine pancreatic acinar cells following administration of different doses of calf-thymus histones in the presence of two different types of heparin: unfractionated heparin and 2-0, 3-0-desulfated (low anticoagulant) heparin. Comparisons by ANOVA, where * denotes $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control and # denotes $p<0.05$ compared to both control and other treatment group. Observations were made in triplicate with $n \geq 4$ experimental repeats.

7.5 The effect of systemic heparin administration depends on the type of heparin used

As the protective effect of heparin pre-treatment has already been investigated, these experiments focus on the potential utility of heparin as a therapy. Therefore, heparin was administered in the familiar 12 injection model of cerulein pancreatitis together with the 4th injection of cerulein – i.e.: 3 hours after induction of pancreatitis. Three different types of heparin were investigated: ODS heparin, UFH and tinzaparin, a type of low molecular weight heparin (LMWH). The common dose of 25 mg/kg has previously been used in the treatment of other inflammatory conditions in murine models (Griffin, Fischer et al. 2014). Figure 7-2 details the therapeutic effects of all three heparins as measured by serum biochemistry, organ protein activity and blinded histology. None of the parameters investigated showed any improvement. In fact, ODS heparin appeared to significantly exacerbate the inflammatory response, producing a statistically significant increase in amylase and IL-6 levels as well as pancreatic and lung myeloperoxidase activity. ODS heparin similarly increased inflammation and necrosis scores, however these differences did not reach statistical significance as the untreated group was already close to the maximal achievable score of 4 points. ODS heparin further led to a statistical and clinically significant difference in pro-inflammatory markers, as well as the only mortality observed by the author in any cerulein-pancreatitis experiments.

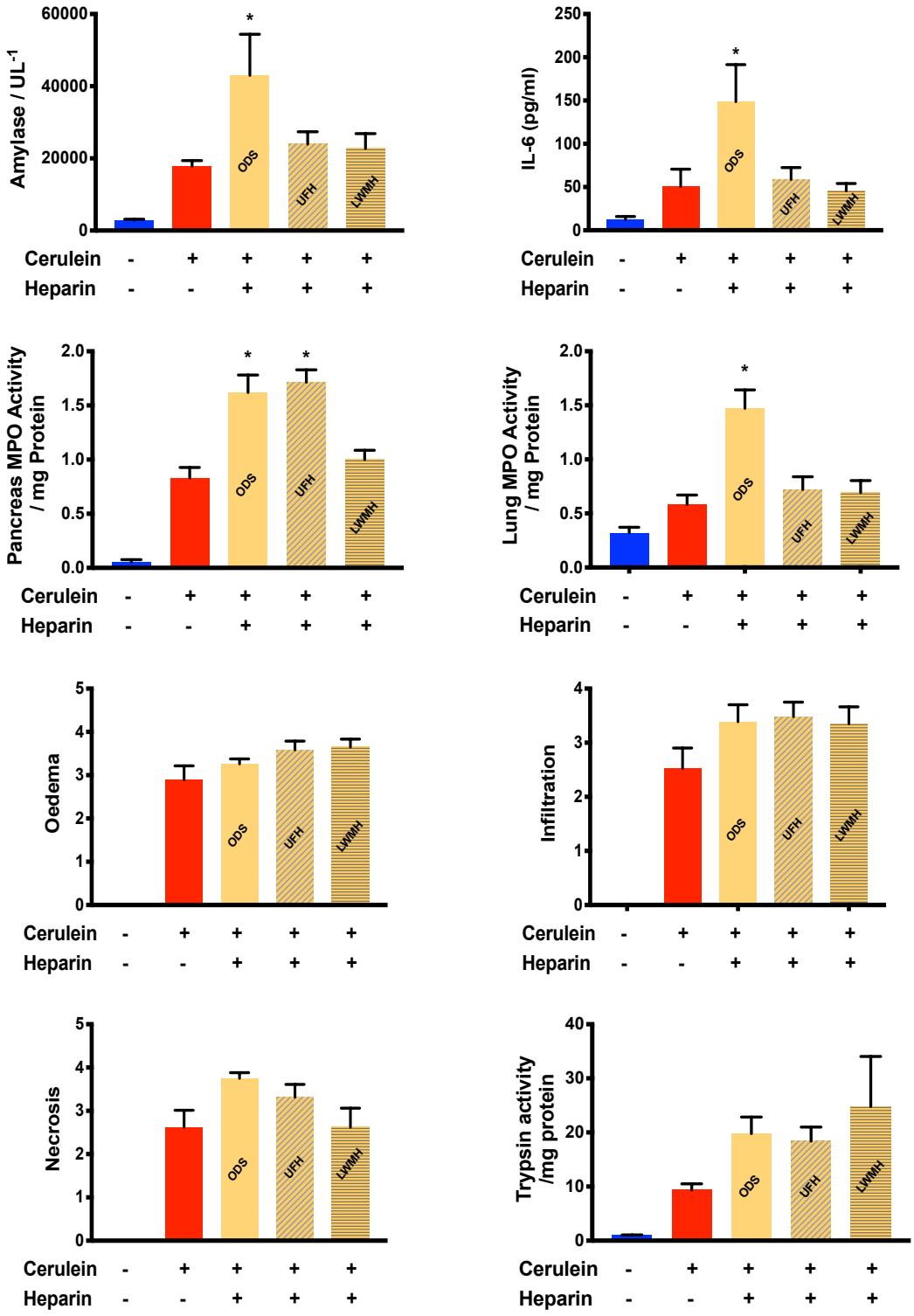


Figure 7-2 Plasma amylase and IL-6 concentration, pancreatic trypsin and pancreatic and lung myeloperoxidase activity as well as blinded histopathological assessment of the pancreas (oedema, infiltration and necrosis) in experimental acute pancreatitis induced by 12x hourly i.p. injections of 50µg/kg cerulein or saline controls. Therapeutic groups all cerulein pancreatitis with three different types of heparin (UFH – 5000 IU/kg, ODS heparin – 25 mg/kg and LMWH – Tinzaparin 25 mg/kg). All comparisons by ANOVA, where * denotes a difference of $p<0.05$ to cerulein; $n \geq 6$.

To investigate this effect further, a lower dose of ODS heparin (10 mg/kg) was tried and the effects on systemic histone release and hepatic injury was measured. Figure 7-3 details the results of these experiments and shows that the lower dose of ODS heparin is not as toxic as the higher dose (but still fails to improve any of the measured parameters of pancreatic injury). Notably, as well as increasing pro-inflammatory mediators of pancreatitis ODS heparin increases levels of circulating histone H3 nearly 3-fold. Similarly, high concentrations of circulating histone H3 have been associated with organ dysfunction and lethal microvascular thromboses, potentially explaining the observed mortality.

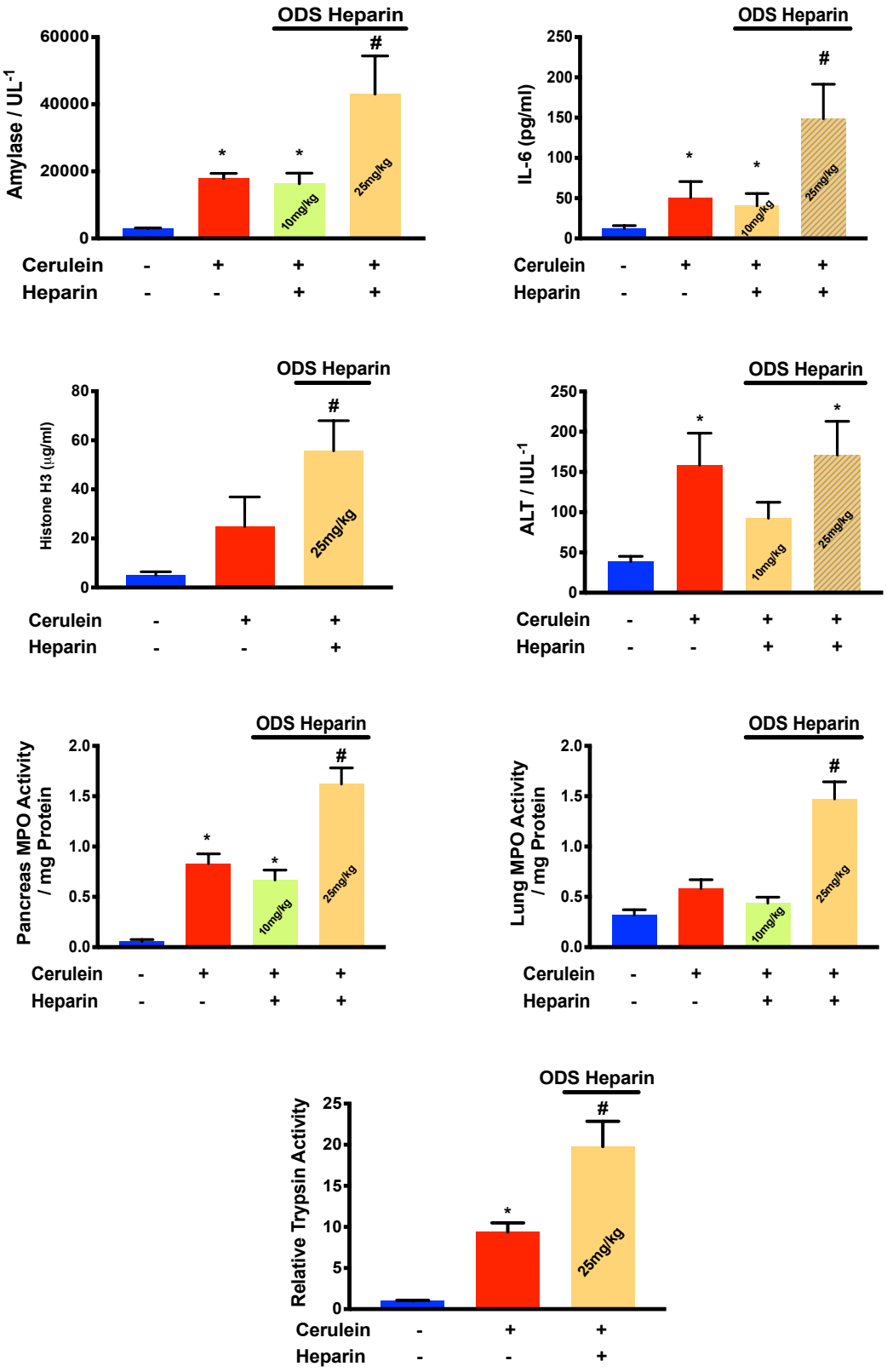


Figure 7-3 Plasma amylase, IL-6, histone H3 and ALT concentration, pancreatic and lung myeloperoxidase activity as well as pancreatic trypsin activity in experimental acute pancreatitis induced by 12x hourly i.p. injections of 50µg/kg cerulein or saline controls. Therapeutic groups all cerulein pancreatitis with low (10 mg/kg) and high (25 mg/kg) doses of the non-anticoagulant ODS heparin. All comparisons by ANOVA, where * denotes a difference of $p<0.05$ to control and # denotes a difference of $p<0.05$ to cerulein; $n \geq 6$.

7.6 Heparins inhibit the degradation of histone H3 in mouse plasma

Histone H3 is rapidly degraded in plasma by the serine protease factor VII-activating protease (FASP). To investigate the kinetics of histone degradation (and thereby detoxification), recombinant histone H3 was spiked into murine plasma at room temperature and incubated for several minutes, hours and overnight, with and without the addition of heparin, At the end of the respective incubation period, the reaction was stopped by heating the solution to above 60 degrees Centigrade and Western blot analysis was undertaken.

Figure 7-4 demonstrates how histone degradation is complete in murine plasma at room temperature in under 10 minutes. Addition of heparin effectively inhibited histone degradation. Although both UFH and ODS heparin inhibited histone degradation, ODS heparin did this more effectively, an effect most evident during the overnight incubation.

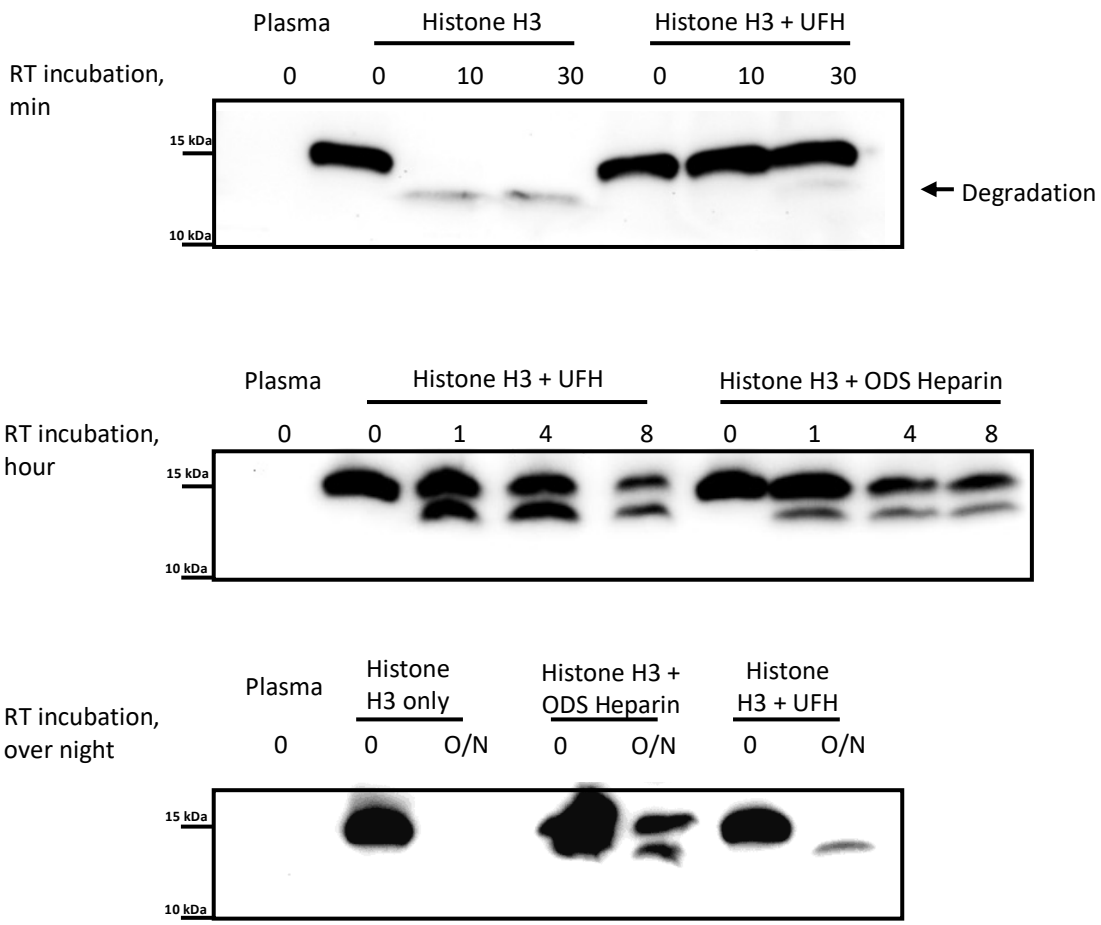


Figure 7-4 Western blot of recombinant histone H3 incubated at room temperature in pooled plasma (C57Bl6/J) and treated with unfractionated (UFH) or non-anticoagulant ODS heparin; n = 3 experimental repeats.

7.7 The effect of systemic heparin administration is dependent on the severity of experimental acute pancreatitis

To investigate whether ODS heparin could similarly exacerbate a milder form of experimental acute pancreatitis, the experiments were repeated using the higher dose (25 mg/kg) of ODS heparin in a 4-cerulein injection model. Figure 7-5 details the standard biochemical and histological parameters. There were no differences observed in any of the measured parameters, including plasma amylase, pancreatic trypsin or myeloperoxidase activity, lung myeloperoxidase activity and blinded histopathological assessment of pancreatic oedema, infiltration and necrosis.

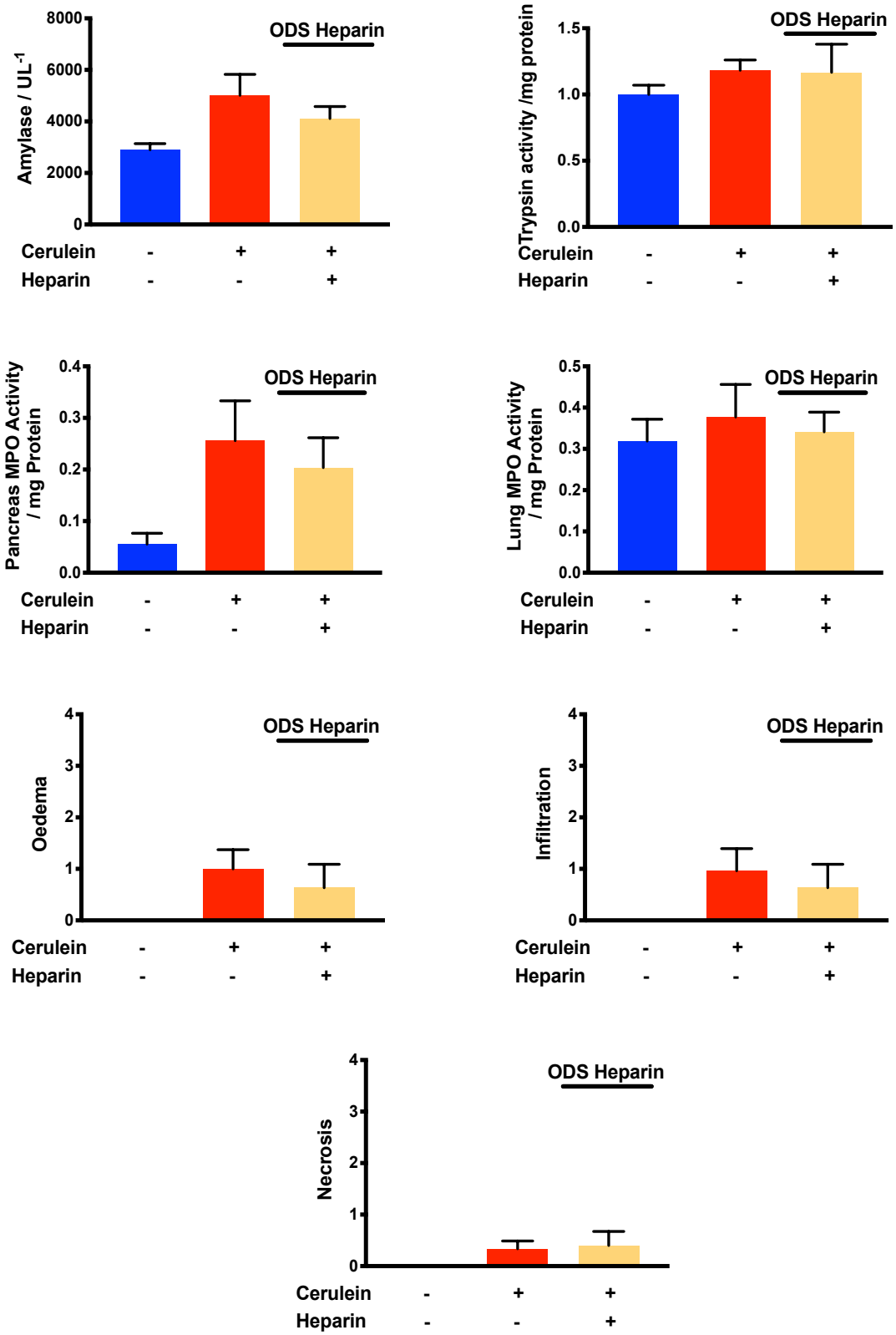


Figure 7-5 Plasma amylase concentration, pancreatic trypsin and pancreatic and lung myeloperoxidase activity as well as blinded histopathological assessment of the pancreas (oedema, infiltration and necrosis) in experimental acute pancreatitis induced by 4x hourly i.p. injections of 50µg/kg cerulein or saline controls. Therapeutic group cerulein pancreatitis with non-anticoagulant ODS heparin (25 mg/kg). All comparisons by ANOVA; n ≥ 6.

7.8 Pre-treatment with an inhibitor of NETosis ameliorates experimental acute pancreatitis

Histone H3 has already been identified as a critical mediator of acute pancreatitis by others and in earlier chapters of this book. Moreover, necrotic acinar cells do not appear to be the primary source of histones in circulation. While it is possible that hepatocyte necrosis contributes to histone release, it is also likely that neutrophil extracellular traps form in hepatic sinusoids as observed during sepsis (McDonald, Urrutia et al. 2012; Tanaka, Koike et al. 2014), and thus contribute to histones measured in the post-hepatic circulation. Therefore, inhibition of NETosis using established and novel inhibitors of PAD4 as pre-treatment should ameliorate acute pancreatitis. The two NETosis-inhibitors tested were Cl-amidine and GSK-484.

Cl-amidine is an irreversible inhibitor of PAD4, with a preference for the Ca^{2+}-bound form of the enzyme (Luo, Arita et al. 2006), whereas GSK-484 was designed to bind reversibly to the low-calcium isoform (Lewis, Liddle et al. 2015). Both effectively inhibit NET-formation *in vitro*. Pre-treatment was chosen, as this should yield the maximally observable benefit. Given the reversible nature of its binding, GSK-484 was given as a split dose and re-administered 4 hours after induction of acute pancreatitis.

Figure 7-6 details the effects of PAD4 inhibition in acute pancreatitis. Trypsin activity, pancreatic and lung myeloperoxidase activity were all improved following administration of Cl-amidine, as were histological assessment of pancreatic oedema, infiltration and necrosis. This coincided with a reduction in plasma histone H3 levels. GSK-484 did improve histopathological parameters, although this did not reach statistical significance. Pancreas and

lung myeloperoxidase activity, amylase levels and trypsin activity were unaffected by GSK-484.

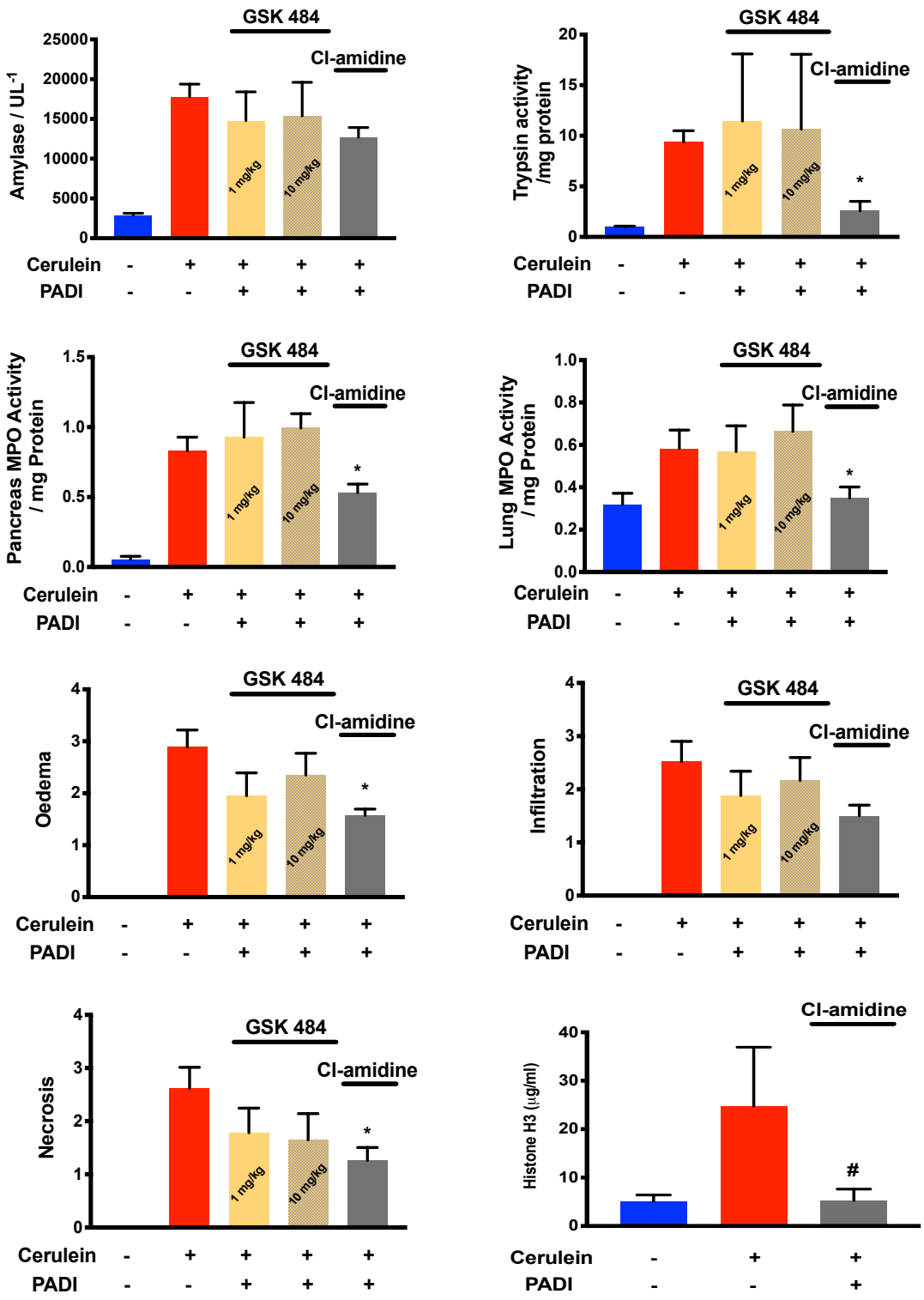


Figure 7-6 Plasma amylase and histone H3 concentrations, pancreatic trypsin and pancreatic and lung myeloperoxidase activity as well as blinded histopathological assessment of the pancreas (oedema, infiltration and necrosis) in experimental acute pancreatitis induced by 12x hourly i.p. injections of 50µg/kg cerulein or saline controls. Therapeutic groups all cerulein pancreatitis, pre-treated with the PAD-4 inhibitors GSK-484 (1 or 10 mg/kg) and Cl-amidine (40 mg/kg). All comparisons by ANOVA, where * denotes a difference of $p<0.05$ to saline and # denotes a difference of $p<0.05$ to cerulein; $n \geq 6$.

7.9 Discussion

Circulating histones are critical to the pathogenesis in acute pancreatitis, as detailed in earlier chapters. Histone toxicity depends on the electrostatic interaction between positively charged histone molecules and negatively charge residues of the plasma membrane. It therefore stands to reason that molecules with a high negative charge density, such as heparin, can detoxify histones. *In vitro* data presented here supports this hypothesis, consistent with data presented in earlier chapters and with published literature (Kleine, Lewis et al. 1997; Szatmary, Liu et al. 2017). *In vivo*, however, this finding is not reproduced using the 12 injection cerulein model. This is contrary to literature suggesting UFH (Ceranowicz, Dembinski et al. 2008) or LMWH (Koksoy, Yankol et al. 2013) are both protective in cerulein-induced pancreatitis. One possible explanation is the timing of therapy, heparin is often administered prior to induction of acute pancreatitis in studies reporting therapeutic success, or type of heparin used. Different heparin fractions can lead to markedly different clinical outcomes, as evidenced by the data using ODS heparin, so it is not reasonable to deduce a response to tinzaparin from other studies using enoxaparin, for example. Using either UFH or tinzaparin as therapy (3 hours after induction of acute pancreatitis), no therapeutic benefit was seen, and no bleeding or other complications observed. Using the low-anticoagulant ODS heparin, however, a marked exacerbation of all parameters could be observed, as well as mortality not usually associated with the cerulein model. This phenomenon requires further explanation.

Firstly, studies using the same ODS heparin obtained from the same source at the same dose have reported inhibition of neutrophil elastase and HMGB1 in

a model of airway inflammation (Griffin, Fischer et al. 2014), both effects should ameliorate acute pancreatitis, not exacerbate it. Secondly, although there was some toxicity observed in *in vitro* studies, no exacerbation of acute pancreatitis was seen in the milder 4 injection model, making substance toxicity an unlikely explanation for this phenomenon. The finding that ODS heparin effectively inhibits histone degradation could be a partial explanation. High concentrations of histones and other positively charged DAMPs such as HMGB1 in the portal circulation could induce a more severe inflammatory response in the liver, and histones in systemic circulation can lead to distal organ dysfunction and worsening pancreas injury as detailed in earlier chapters. UFH, however, also significantly delays histone H3 breakdown, and a similar exacerbation of acute pancreatitis is not observed with UFH. The main difference between ODS heparin and UFH, apart from the relative homogeneity of molecular weight in ODS heparin, is the ability to bind antithrombin (Chen, Lin et al. 2017), which itself has been observed to correlate inversely with histone H3 in sepsis patients (Wildhagen, Wiewel et al. 2015). Therefore, there could well be a confounding effect relating to the specific binding characteristics of ODS heparin, which are not immediately apparent from the data presented here.

With respect to the anticoagulant properties of heparin, something frequently cited as a draw-back when using heparin therapeutically, no bleeding complications were observed during any of the experiments above. Indeed, the role of the coagulation cascade in this context warrants further detailed investigation. Extracellular histones have been observed to interfere with heparins anticoagulant activity (Longstaff, Hogwood et al. 2016), and an

initial period of hypoperfusion and microthrombosis has been reported as beneficial in the context of acute pancreatitis (Ryschich, Kerkadze et al. 2009; Warzecha, Dembinski et al. 2012).

Data on PAD4 inhibition is a little easier to interpret. Cl-amidine effectively reduces extracellular histone levels in acute pancreatitis, together with all other measured markers of inflammation and pancreatic injury. This suggests that the release of histones into systemic circulation is PAD4 dependent and identifies innate immune cells as likely candidates, although it cannot be ruled out that PAD4 inhibition reduces intra-pancreatic injury or affects hepatocytes in a manner that produces the observed experimental results. The finding that GSK-484 is less effective than Cl-amidine has a number of possible explanations. Firstly, GSK-484 has a much lower solubility in water than Cl-amidine and although it does dissolve in aqueous buffer it has the tendency to precipitate, making the effective circulating dose in animals more difficult to predict. Secondly, the fact that GSK-484 binds PAD4 reversibly means that the dosing regimen chosen (2 doses in 12 hours) may still have resulted in lower effective PAD4 inhibition in the *in vivo* setting. Finally, the fact that GSK-484 has a binding preference for the Ca^{2+}-free form of PAD4, whereas Cl-amidine has a preference for the high-Ca^{2+}-bound form is a further possible reason why its therapeutic effects are significantly more pronounced. Its effect on the cell-cycle renders Cl-amidine unsuitable for human use, however the data presented here indicates that the NETosis pathway has promise and warrants further investigation and development.

7.10 Summary

Inhibitors of NETosis ameliorate experimental acute pancreatitis when administered as prophylaxis. Using heparins as systemic therapy in cerulein-induced pancreatitis, despite their ability to bind extracellular histones effectively, was found to be ineffective and potentially detrimental.

8 Overview

The work presented here describes early acute pancreatitis as an inflammatory syndrome, mediated by myeloid cell populations and resulting in systemic inflammation and organ dysfunction. Chapter 3 documents the recruitment of neutrophils and inflammatory monocytes to the injured pancreas by chemokines, as well as the negative feedback systems that limit excessive cellular infiltration. Reduced tissue injury following reduction in myeloid infiltration has been well documented, but data in chapter 3 demonstrate the differential effects of neutrophils and inflammatory monocytes on lung and pancreatic injury. Chapter 4 identifies extracellular histones as critical mediators of pancreatic and systemic injury and the liver as the main source of histones in the systemic circulation. Chapter 5 describes the molecular mechanisms by which histones interact with pancreatic acinar cell membranes, thereby laying the foundations for the genesis of novel therapies. Chapter 6 details the development of novel methods for the investigation of impaired microvascular blood flow in experimental acute pancreatitis, which is a hallmark of immunothrombosis and one of the putative indirect mechanisms of histone-mediated pancreatic injury. Finally, chapter 7 investigates the roles of inhibiting nucleosome release *in vivo* and binding circulating histones using a novel non-anticoagulant heparin as therapeutic agent in the treatment of acute pancreatitis. In this final therapeutic chapter, great care was taken not to replicate well established experimental therapies, so the use of anti-histone antibodies and heparin as a prophylactic agent was avoided.

The findings of the above chapters and how they advance our current understanding of the pathophysiology of early acute pancreatitis is discussed in the subsequent three sections.

8.1 Response to Pancreatic Insult

Investigating the cellular events that occur early (i.e. within minutes to hours) of pancreatic insult necessitates use of experimental models, as patients with acute pancreatitis often present 24-48 hours after the onset of pain (Jin, Lacson et al. 2018). As mentioned earlier, injection of bile into the pancreatic duct of dogs was the first description of an experimental model of acute pancreatitis, and the first documented case of acute pancreatitis of known aetiology in humans by Eugene Opie in 1901 (Lerch, Weidenbach et al. 1994). For the work presented here, however, the hyperstimulation model was chosen rather than the bile-acid model as one that is most reproducible, reliable, best reflects the early events of acute pancreatitis and leads to systemic inflammation entirely resulting from the injury to the pancreas (Gorelick and Lerch 2017). While it is one of the commonest experimental models in use today, it is also one of the least relevant models to human disease. Nevertheless, early immunological mechanisms could not be investigated using a model that requires surgery, which in itself induces inflammation and alters levels of many of the cytokines measured in chapter 3 – inflammatory sequence (Fu, Norman et al. 1996). Moreover, the ability to investigate therapies early following initiation of pancreatitis reliably is an important advantage. Repeated injections of cerulein over several days produces acinar cell loss and pancreatic fibrosis as well as the exocrine and endocrine insufficiency seen in human chronic pancreatitis (Goto, Nakano et

al. 1995; Van Laethem, Robberecht et al. 1996; Murayama, Barent et al. 1999), or indeed recurrent acute pancreatitis, suggesting that the interplay of cerulein-induced pancreatic injury and the immune system has significant similarities with human disease.

The mouse model is also critical in allowing investigation of the extra-pancreatic immunological effects of acute pancreatitis. Chapter 3 details components of the inflammatory cascade that have not been described in this form to date. For example, chemokine expression and release is well described in acinar cells in acute pancreatitis (Saluja and Steer 1999; Sun and Bhatia 2007), however the description of the spacio-temporal relationship is novel. Data in chapter 3 detail chemokine release, leading to amplification of cytokines by the liver and influx of pro-inflammatory monocytes into the pancreas (with a concomitant reduction of monocytes in circulation). The cytokine feedback mechanisms described using cell depletion models had also not been described *in vivo* in acute pancreatitis. Specific depletion of circulating leukocytes is associated with an increase in specific chemokines (i.e. monocytes – MCP-1; neutrophils – KC), without an effect on more generalised inflammatory cytokines (i.e. IL-6, TNFα). Such location-specific mechanisms provide some indication why systemically administered chemokine inhibitors may have limited effects.

The finding that depletion of monocytes appears to limit pancreatic injury, whereas neutrophil depletion limits lung inflammation is in keeping with previous literature, where inhibition of neutrophil elastase using sivelestat protects from lung injury without protection from pancreatic injury (Guo, Yamaguchi et al. 1995; Imamura, Mikami et al. 1998; Wang, Tang et al. 2012)

and monocytes selectively induce pancreatic injury via TNFα (Perides, Weiss et al. 2011). This highlights the importance of being aware of differential effects on similar myeloid cell populations when performing depletion studies, as off-target effects can confound experimental results.

8.2 Systemic Mediators of Pancreatic Inflammatory Syndrome

Acute pancreatitis is recognised as a mild, self-limiting condition in the majority of cases. Patients who suffer mortality do so as a result of catastrophic failure of multiple organ systems, driven by inflammatory mediators. This dichotomy led to the 1992 Atlanta consensus definition of mild and severe acute pancreatitis (Bradley 1993) (revised to include a 'moderate' category in 2012 (Banks, Bollen et al. 2013)) and depends on the presence or absence of systemic disease (organ failure) as well as local complications (pancreatic necrosis and/or fluid collections). Being outcome classifications, patients in the respective categories also exhibit differences in other outcomes such as mortality, length of stay or the need for invasive procedures in the management of their disease.

A large number of physiological and biochemical scoring systems have been developed allowing prediction of these outcomes in retrospective datasets, validating them prospectively with relative accuracy (Khanna, Meher et al. 2013; Vasudevan, Goswami et al. 2018). Chapter 4 demonstrates how these predictors can be supplemented by the measurement of circulating nucleosomes or histones, thereby linking the activity of the innate immune system to the severity of pancreatitis mechanistically. Data presented in

chapter 4 further demonstrate how histone are not only released into the systemic circulation in acute pancreatitis but concentrate within the inflamed pancreas to further exacerbate injury. This dual role of marker of injury severity as well as contributor to further injury (including pancreatic, pulmonary, cardiac and renal (Abrams, Zhang et al. 2013; Bosmann, Grailer et al. 2013; Alhamdi, Zi et al. 2016; Kawai, Kotani et al. 2016)) may explain why histone measurement is at least as good if not better than other markers and can be measured at an earlier time point.

These findings also provide some insight into the genesis of organ failure in the pancreatic inflammatory syndrome. Organ failure is rarely a global phenomenon in acute pancreatitis. The lung is generally the first or only organ to fail (Szatmary, IAP Presentation 2015) and is an independent predictor or mortality (Skouras, Hayes et al. 2014; Parniczky, Kui et al. 2016). While the liver can be seen as the main regulator of local pancreatic versus systemic injury, as indicated in chapter 3 and supported by other pre-clinical data (Gloor, Blinman et al. 2000; Folch-Puy 2007; Wang, Liu et al. 2015), the lung can be injured directly through circulating toxins as well as activated pancreatic proteases bypassing the liver through the thoracic duct in mesenteric lymph (Mittal, Phillips et al. 2009).

Pancreatic necrosis was deemed sufficient to categorize acute pancreatitis as severe in 1992 (Bradley 1993). However, a realisation that necrosis in the absence of organ failure probably did not by itself contribute to a significant increase in mortality (Ashley, Perez et al. 2001) led to a revision of the original Atlanta Classification (Banks, Bollen et al. 2013) which downgraded the significance of pancreatic necrosis in the absence of organ failure and led to

the development of a rival classification system, which eliminated the inclusion of pancreatic necrosis as a determinant of severity entirely (Dellinger, Forsmark et al. 2012). Data presented in chapter 4 support the argument that pancreatic necrosis contributes to organ failure via the pancreatico-hepatic axis, allowing the hypothesis that pancreatic necrosis leads to mortality via organ failure, rather than some intrinsic mechanism. In this model, added mortality seen from infected necrosis (Besselink, van Santvoort et al. 2009; Werge, Novovic et al. 2016) can be in part explained by the increased inflammatory stimulus to the liver and the subsequent increase of systemic inflammation observed in animal models when adding LPS to sterile stimuli (Colletti and Green 2001; Gray, Simovic et al. 2003). This conceptual model of liver-derived systemic inflammation resulting from a co-stimulus of bacterial fragments and DAMPs/cytokines provides a putative explanation why pancreatic necrosis does not lead to organ failure in all cases, and why infected necrosis (Dellinger, Forsmark et al. 2012) and gut barrier dysfunction (Koh, Jeon et al. 2012; Schietroma, Pessia et al. 2016) exacerbates organ failure and mortality in patients with acute pancreatitis.

8.3 Challenges in the Treatment of Acute Pancreatitis

There are a number of practical and pathophysiological challenges in the development of novel therapies for acute pancreatitis. Fundamentally, despite being one of the commonest GI-related reasons for hospital admission in the West, standard trial endpoints such mortality or organ failure are too uncommon to adequately power clinical trials, a problem highlighted in a recent Cochrane review (Moggia, Koti et al. 2017). For example, in an 8-year national dataset study from the US over 69 million hospital discharges were

recorded. This included nearly 1 million patients with acute respiratory distress syndrome, only 32,000 of which were due to acute pancreatitis (Eworuke, Major et al. 2018). That equates to approximately 4000 cases of pancreatitis-related respiratory distress syndrome in the entire US. Problems to adequately power clinical trials in pancreatitis make collaborative efforts between institutions essential. Nevertheless, even large, multinational trials can fail if powered on the wrong outcomes. A prime example of this are the lexipafant trials – a series of randomised controlled trials of a platelet activating factor inhibitor (Kingsnorth, Galloway et al. 1995; McKay, Curran et al. 1997; Wyncoll and Beale 1998; Johnson, Kingsnorth et al. 2001), which were powered on crude endpoints such as mortality and reduction in proportion of complications and included patients up to 72 after onset of the disease and often with established organ failure. The potential of cytokine inhibition lies in prevention of organ failure and, based on data presented in chapter 3, is unlikely to affect pancreatic complications as the cellular inflammatory response in the pancreas is evident by 12 hours after disease onset.

One potential solution to the above problem, is introducing healthcare systems akin to those seen in acute ischaemic events such as myocardial infarction or stroke or indeed sepsis, where early diagnosis and urgent treatment within an hour of onset of symptoms has demonstrable advantages for outcome (Heestermans, van 't Hof et al. 2010; Nugent and Coopersmith 2017). A further solution to the problem of adequate study power lies in selection of robust, continuous outcomes.

8.3.1 Disease activity index

A current development in the management of acute pancreatitis is the measurement of 'disease activity' on a daily basis using a selection of largely clinical parameters to produce a compound activity score. While such a score undoubtedly is of clinical value in its own right, developing such an index would also benefit the development of novel therapeutics as improvements in the continuous activity score variable could be used to power trials. The current leading proponents of a daily disease severity index have published their proposed criteria as the Southern California Pancreas Study Group(Wu, Batech et al. 2017). Their five criteria are any organ failure (as defined by 2 or more points on the Marshall score(Marshall, Cook et al. 1995)), SIRS criteria(Banks, Bollen et al. 2013), abdominal pain, morphine dose in mg and whether or not solid oral diet is tolerated. Experimental data presented in chapter 3 demonstrates how initial pancreatic injury leads to the release of chemokines that recruit inflammatory cells from the bone marrow into the circulation (one of the SIRS criteria) and to the pancreas to contribute to inflammation and pain. Cytokine release is amplified by the liver and contributes to the haemodynamic and pyrogenic effects of systemic inflammation (Yu, Wei et al. 2017). Moreover, IL-1β and TNFα remain elevated 24 hours after induction of pancreatitis and are potent pyrogens and contribute to anorexia of disease (Cannon and Kluger 1983; Zampronio, Melo et al. 1994; Luheshi, Bluthe et al. 2000; Lawrence and Rothwell 2001), supporting the use of SIRS criteria (tachycardia, pyrexia/hypothermia, leucocytosis/leukopoenia, tachypnoea) and return to oral diet as activity indicators.

Data presented in chapters 3 and 4 could aid the development of a biochemical activity score, adding to the understanding of the early cellular and molecular immunological events in the pathogenesis of acute pancreatitis. Indeed many groups have reported on the significance of number and ratios between certain immune cell populations and outcomes in acute pancreatitis (Shrivastava and Bhatia 2010; Zhang, Wu et al. 2016; Jeon and Park 2017) as well as the role of cytokines as disease predictors (Nieminen, Maksimow et al. 2014). Histone release detailed in chapter 4, together with the release of other pro-inflammatory mediators including HMGB1 (Kang, Lotze et al. 2014; Kang, Zhang et al. 2014; Kawai, Kotani et al. 2016) contributes to organ failure in a manner that closely mimics the sequence of organ failure observed in human acute pancreatitis (ie lung before other organs). Therefore, in addition to the clinical manifestations used for the disease activity index one could propose a panel of molecular parameters to supplement the daily activity index including cytokines (TNFα, IL-6, IL1β), histone H3 (and HMGB1) and monocyte/neutrophil numbers to establish a cellular and molecular activity index to objectively probe the pathophysiology and help target therapies to individual patients at the correct time.

8.3.2 Immunothrombosis and drug delivery

Finally, delivering a drug therapy to the injured pancreas is itself fraught with difficulty. For example, inhibitors of pancreatic proteases have been developed and are used in clinical practice, predominantly in Japan, given intra-venously or as continuous regional arterial infusion alone and in conjunction with antibiotics with mixed effects. Despite promise in some

observational studies, larger cohort studies and meta-analyses have shown no benefit (Hamada, Yasunaga et al. 2013; Horibe, Egi et al. 2015; Horibe, Sasaki et al. 2017), and use is not recommended in the latest version of the Japanese guidelines for the management of acute pancreatitis (Yokoe, Takada et al. 2015). Experimentally, gabexate mesilate and nafamostat mesilate (two sereine protease inhibitors in clinical use) infusions improved pancreatic blood flow and increased capillary density in rats with severe acute pancreatitis (Keck, Friebe et al. 2005). This effect, however, has only been demonstrated in concomitant- or pre-treatment. Leukocyte extracellular traps have clot generating and stabilising abilities that make lysis of established clots difficult in the absence of DNAse (Longstaff, Varju et al. 2013; Gould, Vu et al. 2015; Varju, Longstaff et al. 2015; Szatmary, Huang et al. 2018). This 'immunothrombosis' may account for the differential effects seen in protease inhibitor trials. Data in chapter 6 demonstrates the patchy perfusion seen in acute pancreatitis, presumably limiting delivery of therapeutics to areas where they could provide the greatest benefit. Chapter 7 demonstrates the lack of efficacy in treatment – a mere 3 hours after initiation of pancreatitis – of a therapy (heparin) that shows promise when administered prophylactically. Furthermore, while heparin therapy was not effective when administered systemically, possibly in part due to its stabilising effects on circulating histone H3, its ability to bind and reduce injury caused by circulating histones is well demonstrated *in vitro*. Exploring the use of heparin-primed absorption columns in a set-up similar to continuous veno-venous haemofiltration may prove beneficial in early acute pancreatitis, where short-term haemofiltration has already demonstrated a reduction of

organ failure and mortality in patients with severe acute pancreatitis (Guo, Huang et al. 2014; Guo, Suo et al. 2016; Xu, Cui et al. 2017).

Extrapolating from data presented here on the cellular inflammatory sequence and blood flow leads to a view of pancreatitis where injury attracts innate immune cells that enter the pancreas to contain injury by restricting blood supply to the injured area. This then causes further ischaemic injury to adjacent cells. If the volume of pancreas affected is small, resolution of tissue damage occurs. If, however, the segment of under-perfused pancreas is too extensive, the ensuing tissue injury can no longer be contained and the ensuing portal DAMPs induce the liver to augment the systemic inflammatory response. Organ failure and death results. This model is evidently conjecture, however based on data presented in the preceding chapters as well as published literature. To test this hypothesis methods of measuring tissue injury and blood flow *in* vivo and on a whole organ level need to be developed. The MRI-based techniques described in chapter 6 do not provide adequate contrast to image *in* vivo at present, and although detecting perfusion defects is more successful in patients (Yadav, Sharma et al. 2015), linking this finding to portal blood samples is impractical. Nevertheless, if found to broadly hold true, it would mean there are at least two distinct phases of the cellular inflammatory response, firstly containment of injury and secondly control and resolution of cellular damage. This may provide an indication why pancreas-specific therapies have thus far proven difficult to develop and calls for regimen of agents administered at specific stages of the disease to achieve specific goals.

8.3.3 Portal sepsis and the liver as gatekeeper of systemic inflammation

The interaction between the liver, lung and pancreas during acute pancreatitis is under investigated. Chapters 3 and 4 detail how the liver is the source of many cytokines in circulation, in agreement with the literature (Gloor, Todd et al. 1998; Gloor, Blinman et al. 2000). Data in these chapters, however, analyses portal blood in acute pancreatitis, which may represent changes in the proximal gastro-intestinal tract rather than just pancreatic injury. In an elegant study Hoyos et al. demonstrate that systemic inflammation, cytokine levels and lung injury secondary to acute pancreatitis was reduced by creation of a porto-caval shunt, but not a meso-caval shunt(Hoyos, Granell et al. 2005). This indicates not only that the liver is the source of the measured cytokines (TNFα, IL-1β and heat shock protein 72), but that release was induced only by blood from the pancreas and not the proximal gastro-intestinal tract. Nevertheless, intestinal barrier dysfunction and endotoxaemia is a feature of clinical pancreatitis (Schietroma, Pessia et al. 2016; Shen, Li et al. 2017) and occurs maximally between 24 and 48h after disease onset in a rat model of acute pancreatitis (Liang, Chen et al. 2014), a similar time-course to the peak histone release seen in experimental models. LPS is known to exacerbate systemic inflammation when co-administered with cerulein in acute pancreatitis (Ding, Li et al. 2003) and infection of pancreatic necrosis leads to worse outcomes in clinical pancreatitis (Besselink, van Santvoort et al. 2009; Petrov, Shanbhag et al. 2010). This allows the hypothesis that pancreatic injury together with portal sepsis leads to extreme release of inflammatory mediators and fulminant systemic inflammation. It is possible that the gut microbiome contributes to the severity of the disease, as different bacterial species are likely to differ in their ability to translocate across the

injured intestinal mucosa and will stimulate hepatic cytokine release to differing degrees.

8.4 Concluding Remarks

The preceding chapters have described the early cellular events in the inflammatory response to pancreatic injury in acute pancreatitis, some of the feedback mechanisms and have highlighted the critical role of histones as mediators of pancreatic acinar cell death. By beginning to investigate the role of pancreatic blood flow and the porto-hepatic axis as gatekeepers of the pancreatic inflammatory syndrome, new treatment strategies can be developed as well as novel ways to monitor their success.

9 References

Abdulla, A., D. Awla, H. Thorlacius and S. Regner (2011) Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. Journal of Leukocyte Biology.

Abi Abdallah, D. S., C. Lin, C. J. Ball, M. R. King, G. E. Duhamel and E. Y. Denkers (2012) Toxoplasma gondii triggers release of human and mouse neutrophil extracellular traps. Infect Immun 80(2): 768-777.

Abrams, S. T., N. Zhang, C. Dart, S. S. Wang, J. Thachil, Y. Guan, G. Wang and C. H. Toh (2013) Human CRP defends against the toxicity of circulating histones. J Immunol 191(5): 2495-2502.

Abrams, S. T., N. Zhang, J. Manson, T. Liu, C. Dart, F. Baluwa, S. S. Wang, K. Brohi, A. Kipar, W. Yu, G. Wang and C. H. Toh (2013) Circulating histones are mediators of trauma-associated lung injury. AM J RESPIR CRIT CARE MED 187(2): 160-169.

Afghani, E., S. J. Pandol, T. Shimosegawa, R. Sutton, B. U. Wu, S. S. Vege, F. Gorelick, M. Hirota, J. Windsor, S. K. Lo, M. L. Freeman, M. M. Lerch, Y. Tsuji, G. Y. Melmed, W. Wassef and J. Mayerle (2015) Acute Pancreatitis-Progress and Challenges: A Report on an International Symposium. Pancreas 44(8): 1195-1210.

Alhamdi, Y., M. Zi, S. T. Abrams, T. Liu, D. Su, I. Welters, T. Dutt, E. J. Cartwright, G. Wang and C. H. Toh (2016) Circulating Histone Concentrations Differentially Affect the Predominance of Left or Right Ventricular Dysfunction in Critical Illness. Crit Care Med 44(5): e278-288.

Allam, R., S. V. Kumar, M. N. Darisipudi and H. J. Anders (2014) Extracellular histones in tissue injury and inflammation. J Mol Med (Berl) 92(5): 465-472.

Allam, R., C. R. Scherbaum, M. N. Darisipudi, S. R. Mulay, H. Hagele, J. Lichtnekert, J. H. Hagemann, K. V. Rupanagudi, M. Ryu, C. Schwarzenberger, B. Hohenstein, C. Hugo, B. Uhl, C. A. Reichel, F. Krombach, M. Monestier, H. Liapis, K. Moreth, L. Schaefer and H. J. Anders (2012) Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. J Am Soc Nephrol 23(8): 1375-1388.

Ammollo, C. T., F. Semeraro, J. Xu, N. L. Esmon and C. T. Esmon (2011) Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein C activation. J Thromb Haemost 9(9): 1795-1803.

Andrienko, T. N., P. Pasdois, G. C. Pereira, M. J. Ovens and A. P. Halestrap (2017) The role of succinate and ROS in reperfusion injury - A critical appraisal. J Mol Cell Cardiol 110: 1-14.

Arai, Y., Y. Nishinaka, T. Arai, M. Morita, K. Mizugishi, S. Adachi, A. Takaori-Kondo, T. Watanabe and K. Yamashita (2014) Uric acid induces NADPH

oxidase-independent neutrophil extracellular trap formation. Biochem Biophys Res Commun 443(2): 556-561.

Ashley, S. W., A. Perez, E. A. Pierce, D. C. Brooks, F. D. Moore, Jr., E. E. Whang, P. A. Banks and M. J. Zinner (2001) Necrotizing pancreatitis: contemporary analysis of 99 consecutive cases. Ann Surg 234(4): 572-579; discussion 579-580.

Athwal, T., W. Huang, R. Mukherjee, D. Latawiec, M. Chvanov, R. Clarke, K. Smith, F. Campbell, C. Merriman, D. Criddle, R. Sutton, J. Neoptolemos and N. Vlatkovic (2014) Expression of human cationic trypsinogen (PRSS1) in murine acinar cells promotes pancreatitis and apoptotic cell death. Cell Death Dis 5: e1165.

Awla, D., A. Abdulla, I. Syk, B. Jeppsson, S. Regner and H. Thorlacius (2012) Neutrophil-derived matrix metalloproteinase-9 is a potent activator of trypsinogen in acinar cells in acute pancreatitis. Journal of Leukocyte Biology 91(5): 711-719.

Baker, V. S., G. E. Imade, N. B. Molta, P. Tawde, S. D. Pam, M. O. Obadofin, S. A. Sagay, D. Z. Egah, D. Iya, B. B. Afolabi, M. Baker, K. Ford, R. Ford, K. H. Roux and T. C. Keller, 3rd (2008) Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in Plasmodium falciparum infected children under six years of age. Malar J 7: 41.

Banks, P. A., T. L. Bollen, C. Dervenis, H. G. Gooszen, C. D. Johnson, M. G. Sarr, G. G. Tsiotos, S. S. Vege and G. Acute Pancreatitis Classification Working (2013) Classification of acute pancreatitis--2012: revision of the Atlanta classification and definitions by international consensus. Gut 62(1): 102-111.

Banting, F. G., C. H. Best, J. B. Collip, W. R. Campbell and A. A. Fletcher (1922) Pancreatic Extracts in the Treatment of Diabetes Mellitus. Can Med Assoc J 12(3): 141-146.

Barranco-Medina, S., N. Pozzi, A. D. Vogt and E. Di Cera (2013) Histone H4 promotes prothrombin autoactivation. J Biol Chem 288(50): 35749-35757.

Bartneck, M., H. A. Keul, G. Zwadlo-Klarwasser and J. Groll (2010) Phagocytosis independent extracellular nanoparticle clearance by human immune cells. Nano Lett 10(1): 59-63.

Behnen, M., C. Leschczyk, S. Moller, T. Batel, M. Klinger, W. Solbach and T. Laskay (2014) Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. J Immunol 193(4): 1954-1965.

Bell, D. A. and B. Morrison (1991) The spontaneous apoptotic cell death of normal human lymphocytes in vitro: the release of, and immunoproliferative response to, nucleosomes in vitro. Clin Immunol Immunopathol 60(1): 13-26.

Berends, E. T., A. R. Horswill, N. M. Haste, M. Monestier, V. Nizet and M. von Kockritz-Blickwede (2010) Nuclease expression by Staphylococcus aureus

facilitates escape from neutrophil extracellular traps. J Innate Immun 2(6): 576-586.

Bertelli, E., F. Di Gregorio, L. Bertelli, L. Civeli and S. Mosca (1996) The arterial blood supply of the pancreas: a review. II. The posterior superior pancreaticoduodenal artery. An anatomical and radiological study. Surg Radiol Anat 18(1): 1-9.

Bertelli, E., F. Di Gregorio, L. Bertelli, L. Civeli and S. Mosca (1996) The arterial blood supply of the pancreas: a review. III. The inferior pancreaticoduodenal artery. An anatomical review and a radiological study. Surg Radiol Anat 18(2): 67-74.

Bertelli, E., F. Di Gregorio, L. Bertelli and S. Mosca (1995) The arterial blood supply of the pancreas: a review. I. The superior pancreaticoduodenal and the anterior superior pancreaticoduodenal arteries. An anatomical and radiological study. Surg Radiol Anat 17(2): 97-106, 101-103.

Bertelli, E., F. Di Gregorio, L. Bertelli, D. Orazioli and A. Bastianini (1997) The arterial blood supply of the pancreas: a review. IV. The anterior inferior and posterior pancreaticoduodenal aa., and minor sources of blood supply for the head of the pancreas. An anatomical review and radiologic study. Surg Radiol Anat 19(4): 203-212.

Bertelli, E., F. Di Gregorio, S. Mosca and A. Bastianini (1998) The arterial blood supply of the pancreas: a review. V. The dorsal pancreatic artery. An anatomic review and a radiologic study. Surg Radiol Anat 20(6): 445-452.

Besselink, M. G., H. C. van Santvoort, M. A. Boermeester, V. B. Nieuwenhuijs, H. van Goor, C. H. Dejong, A. F. Schaapherder, H. G. Gooszen and G. Dutch Acute Pancreatitis Study (2009) Timing and impact of infections in acute pancreatitis. Br J Surg 96(3): 267-273.

Bhatia, M., A. K. Saluja, B. Hofbauer, H. S. Lee, J. L. Frossard and M. L. Steer (1998) The effects of neutrophil depletion on a completely noninvasive model of acute pancreatitis-associated lung injury. Int J Pancreatol 24(2): 77-83.

Booth, D. M., R. Mukherjee, R. Sutton and D. N. Criddle (2011) Calcium and Reactive Oxygen Species in Acute Pancreatitis: Friend or Foe? Antioxidants & Redox Signaling 15(10): 2683-2698.

Booth, D. M., J. A. Murphy, R. Mukherjee, M. Awais, J. P. Neoptolemos, O. V. Gerasimenko, A. V. Tepikin, O. H. Petersen, R. Sutton and D. N. Criddle (2011) Reactive Oxygen Species Induced by Bile Acid Induce Apoptosis and Protect Against Necrosis in Pancreatic Acinar Cells. Gastroenterology 140(7): 2116-2125.

Bosmann, M., J. J. Grailer, R. Ruemmler, N. F. Russkamp, F. S. Zetoune, J. V. Sarma, T. J. Standiford and P. A. Ward (2013) Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. FASEB J 27(12): 5010-5021.

Bradley, E. L., 3rd (1993) A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. Arch Surg 128(5): 586-590.

Branzk, N., A. Lubojemska, S. E. Hardison, Q. Wang, M. G. Gutierrez, G. D. Brown and V. Papayannopoulos (2014) Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol 15(11): 1017-1025.

Brill, A., T. A. Fuchs, A. S. Savchenko, G. M. Thomas, K. Martinod, S. F. De Meyer, A. A. Bhandari and D. D. Wagner (2012) Neutrophil extracellular traps promote deep vein thrombosis in mice. J Thromb Haemost 10(1): 136-144.

Brinkmann, V. (2004) Neutrophil Extracellular Traps Kill Bacteria. Science 303(5663): 1532-1535.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky (2004) Neutrophil extracellular traps kill bacteria. Science 303(5663): 1532-1535.

Bruns, S., O. Kniemeyer, M. Hasenberg, V. Aimanianda, S. Nietzsche, A. Thywissen, A. Jeron, J. P. Latge, A. A. Brakhage and M. Gunzer (2010) Production of extracellular traps against Aspergillus fumigatus in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. PLoS Pathog 6(4): e1000873.

Buchanan, J. T., A. J. Simpson, R. K. Aziz, G. Y. Liu, S. A. Kristian, M. Kotb, J. Feramisco and V. Nizet (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. Curr Biol 16(4): 396-400.

Cannon, J. G. and M. J. Kluger (1983) Endogenous pyrogen activity in human plasma after exercise. Science 220(4597): 617-619.

Cao, J. and Q. Liu (2013) Protective effects of sivelestat in a caerulein-induced rat acute pancreatitis model. Inflammation 36(6): 1348-1356.

Ceranowicz, P., A. Dembinski, Z. Warzecha, M. Dembinski, J. Cieszkowski, K. Rembisz, S. J. Konturek, B. Kusnierz-Cabala, R. Tomaszewska and W. W. Pawlik (2008) Protective and therapeutic effect of heparin in acute pancreatitis. J Physiol Pharmacol 59 Suppl 4: 103-125.

Chen, G. Y. and G. Nunez (2010) Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 10(12): 826-837.

Chen, H. M., M. Sunamura, K. Shibuya, J. I. Yamauchi, Y. Sakai, S. Fukuyama, Y. Mikami, K. Takeda and S. Matsuno (2001) Early microcirculatory derangement in mild and severe pancreatitis models in mice. Surg Today 31(7): 634-642.

Chen, R., S. Fu, X. G. Fan, M. T. Lotze, H. J. Zeh, 3rd, D. Tang and R. Kang (2015) Nuclear DAMP complex-mediated RAGE-dependent macrophage cell death. Biochem Biophys Res Commun 458(3): 650-655.

Chen, Y., L. Lin, I. Agyekum, X. Zhang, K. St Ange, Y. Yu, F. Zhang, J. Liu, I. J. Amster and R. J. Linhardt (2017) Structural Analysis of Heparin-Derived 3-O-Sulfated Tetrasaccharides: Antithrombin Binding Site Variants. J Pharm Sci 106(4): 973-981.

Chooklin, S., A. Pereyaslov and I. Bihalskyy (2009) Pathogenic role of myeloperoxidase in acute pancreatitis. Hepatobiliary Pancreat Dis Int 8(6): 627-631.

Clark, S. R., A. C. Ma, S. A. Tavener, B. McDonald, Z. Goodarzi, M. M. Kelly, K. D. Patel, S. Chakrabarti, E. McAvoy, G. D. Sinclair, E. M. Keys, E. Allen-Vercoe, R. Devinney, C. J. Doig, F. H. Green and P. Kubes (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med 13(4): 463-469.

Colletti, L. M. and M. Green (2001) Lung and liver injury following hepatic ischemia/reperfusion in the rat is increased by exogenous lipopolysaccharide which also increases hepatic TNF production in vivo and in vitro. Shock 16(4): 312-319.

Cools-Lartigue, J., J. Spicer, B. McDonald, S. Gowing, S. Chow, B. Giannias, F. Bourdeau, P. Kubes and L. Ferri (2013) Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest.

Criddle, D. N., J. V. Gerasimenko, H. K. Baumgartner, M. Jaffar, S. Voronina, R. Sutton, O. H. Petersen and O. V. Gerasimenko (2007) Calcium signalling and pancreatic cell death: apoptosis or necrosis? Cell Death and Differentiation 14(7): 1285-1294.

Criddle, D. N., M. G. Raraty, J. P. Neoptolemos, A. V. Tepikin, O. H. Petersen and R. Sutton (2004) Ethanol toxicity in pancreatic acinar cells: mediation by nonoxidative fatty acid metabolites. Proc Natl Acad Sci U S A 101(29): 10738-10743.

Daley, J. M., A. A. Thomay, M. D. Connolly, J. S. Reichner and J. E. Albina (2008) Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. J Leukoc Biol 83(1): 64-70.

Dawra, R., Y. S. Ku, R. Sharif, D. Dhaulakhandi, P. Phillips, V. Dudeja and A. K. Saluja (2008) An improved method for extracting myeloperoxidase and determining its activity in the pancreas and lungs during pancreatitis. Pancreas 37(1): 62-68.

De Meyer, S. F., G. L. Suidan, T. A. Fuchs, M. Monestier and D. D. Wagner (2012) Extracellular chromatin is an important mediator of ischemic stroke in mice. Arterioscler Thromb Vasc Biol 32(8): 1884-1891.

De Waele, E., E. Wauters, Z. Ling and L. Bouwens (2014) Conversion of human pancreatic acinar cells toward a ductal-mesenchymal phenotype and the role of transforming growth factor beta and activin signaling. Pancreas 43(7): 1083-1092.

Dellinger, E. P., C. E. Forsmark, P. Layer, P. Levy, E. Maravi-Poma, M. S. Petrov, T. Shimosegawa, A. K. Siriwardena, G. Uomo, D. C. Whitcomb, J. A. Windsor, R. Pancreatitis Across Nations Clinical and A. Education (2012) Determinant-based classification of acute pancreatitis severity: an international multidisciplinary consultation. Ann Surg 256(6): 875-880.

Delrue, L., P. Blanckaert, D. Mertens, S. Van Meerbeeck, W. Ceelen and P. Duyck (2012) Tissue perfusion in pathologies of the pancreas: assessment using 128-slice computed tomography. Abdom Imaging 37(4): 595-601.

Deveci, D. and S. Egginton (1999) Development of the fluorescent microsphere technique for quantifying regional blood flow in small mammals. Exp Physiol 84(4): 615-630.

Ding, S. P., J. C. Li and C. Jin (2003) A mouse model of severe acute pancreatitis induced with caerulein and lipopolysaccharide. World J Gastroenterol 9(3): 584-589.

Dolensek, J., M. S. Rupnik and A. Stozer (2015) Structural similarities and differences between the human and the mouse pancreas. Islets 7(1): e1024405.

Donato, M., P. Evelson and R. J. Gelpi (2017) Protecting the heart from ischemia/reperfusion injury: an update on remote ischemic preconditioning and postconditioning. Curr Opin Cardiol 32(6): 784-790.

Drab, T., J. Kracmerova, E. Hanzlikova, T. Cerna, R. Litvakova, A. Pohlova, M. Ticha, P. Prikryl and J. Liberda (2014) The antimicrobial action of histones in the reproductive tract of cow. Biochem Biophys Res Commun 443(3): 987-990.

Dutly, A. E., L. Kugathasan, J. E. Trogadis, S. H. Keshavjee, D. J. Stewart and D. W. Courtman (2006) Fluorescent microangiography (FMA): an improved tool to visualize the pulmonary microvasculature. Lab Invest 86(4): 409-416.

Egan, C. E., W. Sukhumavasi, A. L. Bierly and E. Y. Denkers (2008) Understanding the multiple functions of Gr-1(+) cell subpopulations during microbial infection. Immunol Res 40(1): 35-48.

Eworuke, E., J. M. Major and L. I. Gilbert McClain (2018) National incidence rates for Acute Respiratory Distress Syndrome (ARDS) and ARDS cause-specific factors in the United States (2006-2014). J Crit Care 47: 192-197.

Fattahi, F., J. J. Grailer, L. Jajou, F. S. Zetoune, A. V. Andjelkovic and P. A. Ward (2015) Organ distribution of histones after intravenous infusion of FITC histones or after sepsis. Immunol Res 61(3): 177-186.

Fink, M. P. (2014) Animal models of sepsis. Virulence 5(1): 143-153.

Flowe, K. M., T. H. Welling and M. W. Mulholland (1994) Gastrin-releasing peptide stimulation of amylase release from rat pancreatic lobules involves intrapancreatic neurons. Pancreas 9(4): 513-517.

Foitzik, T., H. G. Hotz, G. Eibl, B. Hotz, M. Kirchengast and H. J. Buhr (1999) Therapy for microcirculatory disorders in severe acute pancreatitis: effectiveness of platelet-activating factor receptor blockade vs. endothelin receptor blockade. J Gastrointest Surg 3(3): 244-251.

Fojo, A. T., P. M. Reuben, P. L. Whitney and W. M. Awad, Jr. (1985) Effect of glycerol on protein acetylation by acetic anhydride. Arch Biochem Biophys 240(1): 43-50.

Folch-Puy, E. (2007) Importance of the liver in systemic complications associated with acute pancreatitis: the role of Kupffer cells. The Journal of Pathology 211(4): 383-388.

Frink, M., H. Andruszkow, C. Zeckey, C. Krettek and F. Hildebrand (2011) Experimental trauma models: an update. J Biomed Biotechnol 2011: 797383.

Frossard, J., A. Saluja, L. Bhagat, H. Lee, M. Bhatia, B. Hofbauer and M. L. Steer (1999) The Role of Intercelullar Adhesion Molecule 1 and Neutrophils in Acute Pancreatitis and Pancreatitis-Associated Lung Injury. Gastroenterology 116: 694-701.

Frossard, J. L., S. Lenglet, F. Montecucco, S. Steffens, K. Galan, G. Pelli, L. Spahr, F. Mach and A. Hadengue (2011) Role of CCL-2, CCR-2 and CCR-4 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. J Clin Pathol 64(5): 387-393.

Frossard, J. L., M. L. Steer and C. M. Pastor (2008) Acute pancreatitis. Lancet 371(9607): 143-152.

Fu, E. S., J. G. Norman, J. E. Scharf and N. Burdash (1996) Effect of type of anesthesia and lower-abdominal laparotomy in mice on the cytokine response to acute stress. Reg Anesth 21(5): 470-473.

Fuchs, T. A., U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann and A. Zychlinsky (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176(2): 231-241.

Fuchs, T. A., A. A. Bhandari and D. D. Wagner (2011) Histones induce rapid and profound thrombocytopenia in mice. Blood 118(13): 3708-3714.

Fuchs, T. A., A. Brill, D. Duerschmied, D. Schatzberg, M. Monestier, D. D. Myers, Jr., S. K. Wrobleski, T. W. Wakefield, J. H. Hartwig and D. D. Wagner (2010) Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 107(36): 15880-15885.

Fuchs, T. A., J. A. Kremer Hovinga, D. Schatzberg, D. D. Wagner and B. Lammle (2012) Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. Blood 120(6): 1157-1164.

Fujimoto, K., R. Hosotani, R. Doi, M. Wada, J. U. Lee, T. Koshiba, Y. Miyamoto and M. Imamura (1997) Role of neutrophils in cerulein-induced pancreatitis in rats: possible involvement of apoptosis. Digestion 58(5): 421-430.

Gamberucci, A., R. Fulceri, P. Marcolongo, W. F. Pralong and A. Benedetti (1998) Histones and basic polypeptides activate Ca2+/cation influx in various cell types. Biochem J 331 (Pt 2): 623-630.

Geiger, S., S. Holdenrieder, P. Stieber, G. F. Hamann, R. Bruening, J. Ma, D. Nagel and D. Seidel (2007) Nucleosomes as a new prognostic marker in early cerebral stroke. J Neurol 254(5): 617-623.

Gerasimenko, J. V., O. Gryshchenko, P. E. Ferdek, E. Stapleton, T. O. Hebert, S. Bychkova, S. Peng, M. Begg, O. V. Gerasimenko and O. H. Petersen (2013) Ca2+ release-activated Ca2+ channel blockade as a potential tool in antipancreatitis therapy. Proc Natl Acad Sci U S A 110(32): 13186-13191.

Gloor, B., T. A. Blinman, D. A. Rigberg, K. E. Todd, J. S. Lane, O. J. Hines and H. A. Reber (2000) Kupffer cell blockade reduces hepatic and systemic cytokine levels and lung injury in hemorrhagic pancreatitis in rats. Pancreas 21(4): 414-420.

Gloor, B., K. E. Todd, J. S. Lane, M. P. Lewis and H. A. Reber (1998) Hepatic Kupffer cell blockade reduces mortality of acute hemorrhagic pancreatitis in mice. J Gastrointest Surg 2(5): 430-435.

Godi, S., B. Eross, Z. Gyomber, A. Szentesi, N. Farkas, A. Parniczky, P. Sarlos, J. Bajor, J. Czimmer, A. Miko, K. Marta, R. Hagendorn, Z. Marton, Z. Verzar, L. Czako, Z. Szepes, A. Vincze and P. Hegyi (2018) Centralized care for acute pancreatitis significantly improves outcomes. J Gastrointestin Liver Dis 27(2): 151-157.

Goodhead, B., H. S. Himal and J. Zanbilowicz (1970) Relationship between pancreatic secretion and pancreatic blood flow. Gut 11(1): 62-68.

Gorelick, F. S. and M. M. Lerch (2017) Do Animal Models of Acute Pancreatitis Reproduce Human Disease? Cell Mol Gastroenterol Hepatol 4(2): 251-262.

Goto, M., I. Nakano, T. Kimura, T. Miyahara, M. Kinjo and H. Nawata (1995) New chronic pancreatitis model with diabetes induced by cerulein plus stress in rats. Dig Dis Sci 40(11): 2356-2363.

Gould, T. J., T. T. Vu, A. R. Stafford, D. J. Dwivedi, P. Y. Kim, A. E. Fox-Robichaud, J. I. Weitz and P. C. Liaw (2015) Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis. Arterioscler Thromb Vasc Biol 35(12): 2544-2553.

Gould, T. J., T. T. Vu, L. L. Swystun, D. J. Dwivedi, S. H. Mai, J. I. Weitz and P. C. Liaw (2014) Neutrophil extracellular traps promote thrombin generation through platelet-dependent and platelet-independent mechanisms. Arterioscler Thromb Vasc Biol 34(9): 1977-1984.

Gray, K. D., M. O. Simovic, W. C. Chapman, T. S. Blackwell, J. W. Christman, A. K. May, K. S. Parman and S. C. Stain (2003) Endotoxin potentiates lung injury in cerulein-induced pancreatitis. Am J Surg 186(5): 526-530.

Gray, R. D., C. D. Lucas, A. Mackellar, F. Li, K. Hiersemenzel, C. Haslett, D. J. Davidson and A. G. Rossi (2013) Activation of conventional protein kinase C

(PKC) is critical in the generation of human neutrophil extracellular traps. J Inflamm (Lond) 10(1): 12.

Griffin, K. L., B. M. Fischer, A. B. Kummarapurugu, S. Zheng, T. P. Kennedy, N. V. Rao, W. M. Foster and J. A. Voynow (2014) 2-O, 3-O-desulfated heparin inhibits neutrophil elastase-induced HMGB-1 secretion and airway inflammation. Am J Respir Cell Mol Biol 50(4): 684-689.

Gukovskaya, A. S., I. Gukovsky, H. Algul and A. Habtezion (2017) Autophagy, Inflammation, and Immune Dysfunction in the Pathogenesis of Pancreatitis. Gastroenterology 153(5): 1212-1226.

Gukovskaya, A. S., I. Gukovsky, V. Zaninovic, M. Song, D. Sandoval, S. Gukovsky and S. J. Pandol (1997) Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. J Clin Invest 100(7): 1853-1862.

Gukovskaya, A. S., E. Vaquero, V. Zaninovic, F. S. Gorelick, A. J. Lusis, M.-L. Brennan, S. Holland and S. J. Pandol (2002) Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. Gastroenterology 122(4): 974-984.

Guo, H., D. W. Suo, H. P. Zhu, X. M. Sun and J. Chen (2016) Early blood purification therapy of severe acute pancreatitis complicated by acute lung injury. Eur Rev Med Pharmacol Sci 20(5): 873-878.

Guo, J., W. Huang, X. N. Yang, T. Jin, K. Altaf, M. A. Javed, Z. Q. Lin, Z. W. Huang, P. Xue, M. Johnstone, R. Sutton and Q. Xia (2014) Short-term continuous high-volume hemofiltration on clinical outcomes of severe acute pancreatitis. Pancreas 43(2): 250-254.

Guo, L., Y. Yamaguchi, S. Ikei, H. Sugita and M. Ogawa (1995) Neutrophil Elastase Inhibitor (ONO-5046) Prevents Lung Hemorrhage Induced by Lipopolysaccharide in Rat Model of Cerulein Pancreatitis. Digestive Diseases and Sciences 40(10): 2177-2183.

Habtezion, A. (2015) Inflammation in acute and chronic pancreatitis. Curr Opin Gastroenterol 31(5): 395-399.

Habtezion, A. and H. Algul (2016) Immune modulation in acute and chronic pancreatitis. Pancreapedia: Exocrine Pancreas Knowledge Base.

Hackert, T. and M. W. Buchler (2016) Decision Making in Necrotizing Pancreatitis. Dig Dis 34(5): 517-524.

Hackert, T., D. Pfeil, W. Hartwig, S. Fritz, L. Schneider, M. M. Gebhard, M. W. Buchler and J. Werner (2007) Platelet function in acute experimental pancreatitis. J Gastrointest Surg 11(4): 439-444.

Hall, P. A. and N. R. Lemoine (1992) Rapid acinar to ductal transdifferentiation in cultured human exocrine pancreas. J Pathol 166(2): 97-103.

Hamada, T., H. Yasunaga, Y. Nakai, H. Isayama, H. Horiguchi, S. Matsuda, K. Fushimi and K. Koike (2013) Continuous regional arterial infusion for acute pancreatitis: a propensity score analysis using a nationwide administrative database. Crit Care 17(5): R214.

Heestermans, T., A. W. van 't Hof, J. M. ten Berg, J. W. van Werkum, E. Boersma, A. Mosterd, P. R. Stella, A. B. van Zoelen, A. T. Gosselink, W. Kochman, T. Dill, P. C. Koopmans, G. van Houwelingen, F. Zijlstra and C. Hamm (2010) The golden hour of prehospital reperfusion with triple antiplatelet therapy: a sub-analysis from the Ongoing Tirofiban in Myocardial Evaluation 2 (On-TIME 2) trial early initiation of triple antiplatelet therapy. Am Heart J 160(6): 1079-1084.

Hegyi, P., M. Wilschanski, S. Muallem, G. L. Lukacs, M. Sahin-Toth, A. Uc, M. A. Gray, Z. Rakonczay, Jr. and J. Maleth (2016) CFTR: A New Horizon in the Pathomechanism and Treatment of Pancreatitis. Rev Physiol Biochem Pharmacol 170: 37-66.

Hirose, T., S. Hamaguchi, N. Matsumoto, T. Irisawa, M. Seki, O. Tasaki, H. Hosotsubo, N. Yamamoto, K. Yamamoto, Y. Akeda, K. Oishi, K. Tomono and T. Shimazu (2014) Presence of neutrophil extracellular traps and citrullinated histone H3 in the bloodstream of critically ill patients. PLoS One 9(11): e111755.

Hoque, R., A. F. Malik, F. Gorelick and W. Z. Mehal (2012) Sterile inflammatory response in acute pancreatitis. Pancreas 41(3): 353-357.

Horibe, M., M. Egi, M. Sasaki and M. Sanui (2015) Continuous Regional Arterial Infusion of Protease Inhibitors for Treatment of Severe Acute Pancreatitis: Systematic Review and Meta-Analysis. Pancreas 44(7): 1017-1023.

Horibe, M., M. Sasaki, M. Sanui, D. Sugiyama, E. Iwasaki, Y. Yamagishi, H. Sawano, T. Goto, T. Ikeura, T. Hamada, T. Oda, H. Yasuda, W. Shinomiya, D. Miyazaki, K. Hirose, K. Kitamura, N. Chiba, T. Ozaki, T. Yamashita, T. Koinuma, T. Oshima, T. Yamamoto, M. Hirota, T. Moriya, K. Shirai, T. Mayumi and T. Kanai (2017) Continuous Regional Arterial Infusion of Protease Inhibitors Has No Efficacy in the Treatment of Severe Acute Pancreatitis: A Retrospective Multicenter Cohort Study. Pancreas 46(4): 510-517.

Hoyos, S., S. Granell, N. Heredia, O. Bulbena, D. Closa and L. Fernández-Cruz (2005) Influence of portal blood on the development of systemic inflammation associated with experimental acute pancreatitis. Surgery 137(2): 186-191.

Huang, H., H. W. Chen, J. Evankovich, W. Yan, B. R. Rosborough, G. W. Nace, Q. Ding, P. Loughran, D. Beer-Stolz, T. R. Billiar, C. T. Esmon and A. Tsung (2013) Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. J Immunol 191(5): 2665-2679.

Huang, H., S. Tohme, A. B. Al-Khafaji, S. Tai, P. Loughran, L. Chen, S. Wang, J. Kim, T. Billiar, Y. Wang and A. Tsung (2015) Damage-associated molecular pattern-activated neutrophil extracellular trap exacerbates sterile inflammatory liver injury. Hepatology 62(2): 600-614.

Huang, W., D. M. Booth, M. C. Cane, M. Chvanov, M. A. Javed, V. L. Elliott, J. A. Armstrong, H. Dingsdale, N. Cash, Y. Li, W. Greenhalf, R. Mukherjee, B. S. Kaphalia, M. Jaffar, O. H. Petersen, A. V. Tepikin, R. Sutton and D. N. Criddle (2014) Fatty acid ethyl ester synthase inhibition ameliorates ethanol-induced Ca2+-dependent mitochondrial dysfunction and acute pancreatitis. Gut 63(8): 1313-1324.

Huang, W., M. C. Cane, R. Mukherjee, P. Szatmary, X. Zhang, V. Elliott, Y. Ouyang, M. Chvanov, D. Latawiec, L. Wen, D. M. Booth, A. C. Haynes, O. H. Petersen, A. V. Tepikin, D. N. Criddle and R. Sutton (2015) Caffeine protects against experimental acute pancreatitis by inhibition of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ release. Gut.

Huang, W., M. C. Cane, R. Mukherjee, P. Szatmary, X. Zhang, V. Elliott, Y. Ouyang, M. Chvanov, D. Latawiec, L. Wen, D. M. Booth, A. C. Haynes, O. H. Petersen, A. V. Tepikin, D. N. Criddle and R. Sutton (2017) Caffeine protects against experimental acute pancreatitis by inhibition of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ release. Gut 66(2): 301-313.

Huang, W., N. Cash, L. Wen, P. Szatmary, R. Mukherjee, J. Armstrong, M. Chvanov, A. V. Tepikin, M. P. Murphy, R. Sutton and D. N. Criddle (2015) Effects of the mitochondria-targeted antioxidant mitoquinone in murine acute pancreatitis. Mediators Inflamm 2015: 901780.

Huang, Y. C., H. Y. Gaisano and Y. M. Leung (2011) Electrophysiological identification of mouse islet alpha-cells: from isolated single alpha-cells to in situ assessment within pancreas slices. Islets 3(4): 139-143.

IAP/APA Working Group (2013) IAP/APA evidence-based guidelines for the management of acute pancreatitis. Pancreatology 13(4 Suppl 2): e1-15.

Iba, T., S. Gando and J. Thachil (2014) Anticoagulant therapy for sepsis-associated disseminated intravascular coagulation: the view from Japan. J Thromb Haemost 12(7): 1010-1019.

Ikeura, T., K. Kato, M. Takaoka, M. Shimatani, M. Kishimoto, K. Nishi, S. Kariya and K. Okazaki (2017) A body mass index >/=25 kg/m(2) is associated with a poor prognosis in patients with acute pancreatitis: a study of Japanese patients. Hepatobiliary Pancreat Dis Int 16(6): 645-651.

Imamura, M., Y. Mikami, H. Takahashi and H. Yamauchi (1998) Effect of a specific synthetic inhibitor of neutrophil elastase (ONO-5046) on the course of acute hemorrhagic pancreatitis in dogs. J Hepatobiliary Pancreat Surg 5(4): 422-428.

Inoue, S., A. Nakao, W. Kishimoto, H. Murakami, K. Itoh, T. Itoh, A. Harada, T. Nonami and H. Takagi (1995) Anti-neutrophil antibody attenuates the severity of acute lung injury in rats with experimental acute pancreatitis. Arch Surg 130(1): 93-98.

Iskander, K. N., M. F. Osuchowski, D. J. Stearns-Kurosawa, S. Kurosawa, D. Stepien, C. Valentine and D. G. Remick (2013) Sepsis: multiple abnormalities,

heterogeneous responses, and evolving understanding. Physiol Rev 93(3): 1247-1288.

Jeon, T. J. and J. Y. Park (2017) Clinical significance of the neutrophil-lymphocyte ratio as an early predictive marker for adverse outcomes in patients with acute pancreatitis. World J Gastroenterol 23(21): 3883-3889.

Jhunjhunwala, S., S. Aresta-DaSilva, K. Tang, D. Alvarez, M. J. Webber, B. C. Tang, D. M. Lavin, O. Veiseh, J. C. Doloff, S. Bose, A. Vegas, M. Ma, G. Sahay, A. Chiu, A. Bader, E. Langan, S. Siebert, J. Li, D. L. Greiner, P. E. Newburger, U. H. von Andrian, R. Langer and D. G. Anderson (2015) Neutrophil Responses to Sterile Implant Materials. PLoS One 10(9): e0137550.

Jin, D. X., R. Lacson, L. R. Cochon, E. C. Alper, J. McNabb-Baltar, P. A. Banks and R. Khorasani (2018) A Clinical Model for the Early Diagnosis of Acute Pancreatitis in the Emergency Department. Pancreas 47(7): 871-879.

Johnson, C. D., A. N. Kingsnorth, C. W. Imrie, M. J. McMahon, J. P. Neoptolemos, C. McKay, S. K. Toh, P. Skaife, P. C. Leeder, P. Wilson, M. Larvin and L. D. Curtis (2001) Double blind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. Gut 48(1): 62-69.

Juneau, R. A., B. Pang, K. E. Weimer, C. E. Armbruster and W. E. Swords (2011) Nontypeable Haemophilus influenzae initiates formation of neutrophil extracellular traps. Infect Immun 79(1): 431-438.

Kadiyala, V., L. S. Lee, P. A. Banks, S. Suleiman, J. A. Paulo, W. Wang, J. Rosenblum, N. I. Sainani, K. Mortele and D. L. Conwell (2013) Cigarette smoking impairs pancreatic duct cell bicarbonate secretion. JOP 14(1): 31-38.

Kalbitz, M., J. J. Grailer, F. Fattahi, L. Jajou, T. J. Herron, K. F. Campbell, F. S. Zetoune, M. Bosmann, J. V. Sarma, M. Huber-Lang, F. Gebhard, R. Loaiza, H. H. Valdivia, J. Jalife, M. W. Russell and P. A. Ward (2015) Role of extracellular histones in the cardiomyopathy of sepsis. FASEB J 29(5): 2185-2193.

Kambhampati, S., W. Park and A. Habtezion (2014) Pharmacologic therapy for acute pancreatitis. World J Gastroenterol 20(45): 16868-16880.

Kang, R., M. T. Lotze, H. J. Zeh, T. R. Billiar and D. Tang (2014) Cell death and DAMPs in acute pancreatitis. Mol Med 20: 466-477.

Kang, R., Q. Zhang, W. Hou, Z. Yan, R. Chen, J. Bonaroti, P. Bansal, T. R. Billiar, A. Tsung, Q. Wang, D. L. Bartlett, D. C. Whitcomb, E. B. Chang, X. Zhu, H. Wang, B. Lu, K. J. Tracey, L. Cao, X. G. Fan, M. T. Lotze, H. J. Zeh, 3rd and D. Tang (2014) Intracellular Hmgb1 inhibits inflammatory nucleosome release and limits acute pancreatitis in mice. Gastroenterology 146(4): 1097-1107.

Kawai, C., H. Kotani, M. Miyao, T. Ishida, L. Jemail, H. Abiru and K. Tamaki (2016) Circulating Extracellular Histones Are Clinically Relevant Mediators of Multiple Organ Injury. Am J Pathol 186(4): 829-843.

Ke, L., H. B. Ni, Z. H. Tong, W. Q. Li, N. Li and J. S. Li (2014) Efficacy of continuous regional arterial infusion with low-molecular-weight heparin for severe acute pancreatitis in a porcine model. Shock 41(5): 443-448.

Keck, T., V. Friebe, A. L. Warshaw, B. A. Antoniu, G. Waneck, S. Benz, U. T. Hopt and C. Fernandez-del-Castillo (2005) Pancreatic proteases in serum induce leukocyte-endothelial adhesion and pancreatic microcirculatory failure. Pancreatology 5(2-3): 241-250.

Keminger, K. (2001). Pankreas. Viszeralchirurgie. W. Hartel, K. Keminger, M. Rehner, H.-B. Reith and H. W. Schreiber. EU, Einhorn-Presse Verlag**:** 403-422.

Keshari, R. S., A. Jyoti, M. Dubey, N. Kothari, M. Kohli, J. Bogra, M. K. Barthwal and M. Dikshit (2012) Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One 7(10): e48111.

Khanna, A. K., S. Meher, S. Prakash, S. K. Tiwary, U. Singh, A. Srivastava and V. K. Dixit (2013) Comparison of Ranson, Glasgow, MOSS, SIRS, BISAP, APACHE-II, CTSI Scores, IL-6, CRP, and Procalcitonin in Predicting Severity, Organ Failure, Pancreatic Necrosis, and Mortality in Acute Pancreatitis. HPB Surg 2013: 367581.

Kheiri, S. A., T. M. Fasy and H. H. Billett (1996) Effects of H1 histones and a monoclonal autoantibody to H1 histones on clot formation in vitro: possible implications in the antiphospholipid syndrome. Thromb Res 82(1): 43-50.

Kim, D. B., W. C. Chung, J. M. Lee, K. M. Lee, J. H. Oh and E. J. Jeon (2017) Analysis of Factors Associated with the Severity of Acute Pancreatitis according to Etiology. Gastroenterol Res Pract 2017: 1219464.

Kim, H. S., J. H. Cho, H. W. Park, H. Yoon, M. S. Kim and S. C. Kim (2002) Endotoxin-neutralizing antimicrobial proteins of the human placenta. J Immunol 168(5): 2356-2364.

Kim, J. E., N. Lee, J. Y. Gu, H. J. Yoo and H. K. Kim (2015) Circulating levels of DNA-histone complex and dsDNA are independent prognostic factors of disseminated intravascular coagulation. Thromb Res 135(6): 1064-1069.

Kingsnorth, A. N., S. W. Galloway and L. J. Formela (1995) Randomized, double-blind phase II trial of Lexipafant, a platelet-activating factor antagonist, in human acute pancreatitis. Br J Surg 82(10): 1414-1420.

Kirchner, T., S. Moller, M. Klinger, W. Solbach, T. Laskay and M. Behnen (2012) The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. Mediators Inflamm 2012: 849136.

Kleine, T. J., P. N. Lewis and S. A. Lewis (1997) Histone-induced damage of a mammalian epithelium: the role of protein and membrane structure. Am J Physiol 273(6 Pt 1): C1925-1936.

Koh, Y. Y., W. K. Jeon, Y. K. Cho, H. J. Kim, W. G. Chung, C. U. Chon, T. Y. Oh and J. H. Shin (2012) The effect of intestinal permeability and endotoxemia on the prognosis of acute pancreatitis. Gut Liver 6(4): 505-511.

Koksoy, F. N., Y. Yankol, E. Sen Oran, S. Ozkan Gurdal, M. Yuksel, A. Akyildiz Igdem, N. Yildirim Yazgan and G. R. Soybir (2013) Preventive effects of enoxaparin and hesperidin in cerulein-induced acute pancreatitis in rats. Turk J Gastroenterol 24(6): 495-501.

Kolaczkowska, E., C. N. Jenne, B. G. Surewaard, A. Thanabalasuriar, W. Y. Lee, M. J. Sanz, K. Mowen, G. Opdenakker and P. Kubes (2015) Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. Nat Commun 6: 6673.

Koo, Y. S., J. M. Kim, I. Y. Park, B. J. Yu, S. A. Jang, K. S. Kim, C. B. Park, J. H. Cho and S. C. Kim (2008) Structure-activity relations of parasin I, a histone H2A-derived antimicrobial peptide. Peptides 29(7): 1102-1108.

Korhonen, J. T., V. Dudeja, R. Dawra, P. Kubes and A. Saluja (2015) Neutrophil Extracellular Traps Provide a Grip on the Enigmatic Pathogenesis of Acute Pancreatitis. Gastroenterology 149(7): 1682-1685.

Kossel, A. (1884) Ueber einen peptonartigen Bestandteil des Zellkerns. Zschr physiol Chem 8: 511-515.

Kouzarides, T. (2007) Chromatin modifications and their function. Cell 128(4): 693-705.

Koyasu, S., H. Isoda, Y. Tsuji, H. Yamamoto, K. Matsueda, Y. Watanabe, T. Chiba and K. Togashi (2012) Hepatic arterial perfusion increases in the early stage of severe acute pancreatitis patients: evaluation by perfusion computed tomography. Eur J Radiol 81(1): 43-46.

Kumar, A., D. Roberts, K. E. Wood, B. Light, J. E. Parrillo, S. Sharma, R. Suppes, D. Feinstein, S. Zanotti, L. Taiberg, D. Gurka, A. Kumar and M. Cheang (2006) Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med 34(6): 1589-1596.

Kusske, A. M., A. J. Rongione, S. W. Ashley, D. W. McFadden and H. A. Reber (1996) Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. Surgery 120(2): 284-288; discussion 289.

Kutcher, M. E., J. Xu, R. F. Vilardi, C. Ho, C. T. Esmon and M. J. Cohen (2012) Extracellular histone release in response to traumatic injury: implications for a compensatory role of activated protein C. J Trauma Acute Care Surg 73(6): 1389-1394.

Lawrence, C. B. and N. J. Rothwell (2001) Anorexic but not pyrogenic actions of interleukin-1 are modulated by central melanocortin-3/4 receptors in the rat. J Neuroendocrinol 13(6): 490-495.

Lerch, M. M., H. Weidenbach, C. A. Hernandez, G. Preclik and G. Adler (1994) Pancreatic outflow obstruction as the critical event for human gall stone induced pancreatitis. Gut 35(10): 1501-1503.

Leshner, M., S. Wang, C. Lewis, H. Zheng, X. A. Chen, L. Santy and Y. Wang (2012) PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. Front Immunol 3: 307.

Lete, M. G., J. Sot, H. Ahyayauch, N. Fernandez-Rivero, A. Prado, F. M. Goni and A. Alonso (2014) Histones and DNA compete for binding polyphosphoinositides in bilayers. Biophys J 106(5): 1092-1100.

Lewis, H. D., J. Liddle, J. E. Coote, S. J. Atkinson, M. D. Barker, B. D. Bax, K. L. Bicker, R. P. Bingham, M. Campbell, Y. H. Chen, C. W. Chung, P. D. Craggs, R. P. Davis, D. Eberhard, G. Joberty, K. E. Lind, K. Locke, C. Maller, K. Martinod, C. Patten, O. Polyakova, C. E. Rise, M. Rudiger, R. J. Sheppard, D. J. Slade, P. Thomas, J. Thorpe, G. Yao, G. Drewes, D. D. Wagner, P. R. Thompson, R. K. Prinjha and D. M. Wilson (2015) Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. Nat Chem Biol 11(3): 189-191.

Lewis, M. P., H. A. Reber and S. W. Ashley (1998) Pancreatic blood flow and its role in the pathophysiology of pancreatitis. J Surg Res 75(1): 81-89.

Li, J. J. and E. J. Corey (2013). Chapter 5: Cardiovascular Drugs. Drug Discovery: Practices, Processes, and Perspectives. J. J. Li and E. J. Corey. Hoboken, New Jersey, John Wiley & Sons Inc: 189-190.

Liang, H. Y., T. Chen, T. Wang, Z. Huang, H. T. Yan and L. J. Tang (2014) Time course of intestinal barrier function injury in a sodium taurocholate-induced severe acute pancreatitis in rat model. J Dig Dis 15(7): 386-393.

Liu, C. L., S. Tangsombatvisit, J. M. Rosenberg, G. Mandelbaum, E. C. Gillespie, O. P. Gozani, A. A. Alizadeh and P. J. Utz (2012) Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies. Arthritis Res Ther 14(1): R25.

Liu, H. B., N. Q. Cui, D. H. Li and C. Chen (2006) Role of Kupffer cells in acute hemorrhagic necrotizing pancreatitis-associated lung injury of rats. World J Gastroenterol 12(3): 403-407.

Liu, T., W. Huang, P. Szatmary, S. T. Abrams, Y. Alhamdi, Z. Lin, W. Greenhalf, G. Wang, R. Sutton and C. H. Toh (2017) Accuracy of circulating histones in predicting persistent organ failure and mortality in patients with acute pancreatitis. Br J Surg.

Liu, X. Y., L. Xue, X. Zheng, S. Yan and S. S. Zheng (2010) Pancreas transplantation in the mouse. Hepatobiliary Pancreat Dis Int 9(3): 254-258.

Liu, Z. G., S. Y. Ni, G. M. Chen, J. Cai, Z. H. Guo, P. Chang and Y. S. Li (2013) Histones-mediated lymphocyte apoptosis during sepsis is dependent on p38

phosphorylation and mitochondrial permeability transition. PLoS One 8(10): e77131.

Longstaff, C., J. Hogwood, E. Gray, E. Komorowicz, I. Varju, Z. Varga and K. Kolev (2016) Neutralisation of the anti-coagulant effects of heparin by histones in blood plasma and purified systems. Thromb Haemost 115(3): 591-599.

Longstaff, C., I. Varju, P. Sotonyi, L. Szabo, M. Krumrey, A. Hoell, A. Bota, Z. Varga, E. Komorowicz and K. Kolev (2013) Mechanical stability and fibrinolytic resistance of clots containing fibrin, DNA, and histones. J Biol Chem 288(10): 6946-6956.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent and T. J. Richmond (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389(6648): 251-260.

Luheshi, G. N., R. M. Bluthe, D. Rushforth, N. Mulcahy, J. P. Konsman, M. Goldbach and R. Dantzer (2000) Vagotomy attenuates the behavioural but not the pyrogenic effects of interleukin-1 in rats. Auton Neurosci 85(1-3): 127-132.

Lundberg, A. H., J. W. Eubanks, 3rd, J. Henry, O. Sabek, M. Kotb, L. Gaber, A. Norby-Teglund and A. O. Gaber (2000) Trypsin stimulates production of cytokines from peritoneal macrophages in vitro and in vivo. Pancreas 21(1): 41-51.

Luo, Y., K. Arita, M. Bhatia, B. Knuckley, Y. H. Lee, M. R. Stallcup, M. Sato and P. R. Thompson (2006) Inhibitors and inactivators of protein arginine deiminase 4: functional and structural characterization. Biochemistry 45(39): 11727-11736.

Lur, G., M. Sherwood, E. Ebisui, L. Haynes, S. Feske, R. Sutton, R. Burgoyne, K. Mikoshiba, O. Petersen and A. Tepikin (2011) IP3 receptors and Orai channels in pancreatic acinar cells: co-localisation and its consequences. Biochemical Journal.

Lv, X., T. Wen, J. Song, D. Xie, L. Wu, X. Jiang, P. Jiang and Z. Wen (2017) Extracellular histones are clinically relevant mediators in the pathogenesis of acute respiratory distress syndrome. Respir Res 18(1): 165.

Macchi, V., E. E. E. Picardi, A. Porzionato, A. Morra, R. Bardini, M. Loukas, R. S. Tubbs and R. De Caro (2017) Anatomo-radiological patterns of pancreatic vascularization, with surgical implications: Clinical and anatomical study. Clin Anat 30(5): 614-624.

Majewski, P., M. Majchrzak-Gorecka, B. Grygier, J. Skrzeczynska-Moncznik, O. Osiecka and J. Cichy (2016) Inhibitors of Serine Proteases in Regulating the Production and Function of Neutrophil Extracellular Traps. Front Immunol 7: 261.

Malla, S. R., C. Karrman Mardh, A. Gunther, U. M. Mahajan, M. Sendler, J. D'Haese, F. U. Weiss, M. M. Lerch, M. B. Hansen and J. Mayerle (2016) Effect of

oral administration of AZD8309, a CXCR2 antagonist, on the severity of experimental pancreatitis. Pancreatology 16(5): 761-769.

Mandalia, A., E. J. Wamsteker and M. DiMagno (2018) Recent advances in understanding and managing acute pancreatitis. F1000Res 7.

Mandelbaum, I. and C. R. Morgan (1969) Pancreatic blood flow and its relationship to insulin secretion during extracorporeal circulation. Ann Surg 170(5): 753-758.

Marcos, V., Z. Zhou, A. O. Yildirim, A. Bohla, A. Hector, L. Vitkov, E. M. Wiedenbauer, W. D. Krautgartner, W. Stoiber, B. H. Belohradsky, N. Rieber, M. Kormann, B. Koller, A. Roscher, D. Roos, M. Griese, O. Eickelberg, G. Doring, M. A. Mall and D. Hartl (2010) CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. Nat Med 16(9): 1018-1023.

Marshall, J. C., D. J. Cook, N. V. Christou, G. R. Bernard, C. L. Sprung and W. J. Sibbald (1995) Multiple organ dysfunction score: a reliable descriptor of a complex clinical outcome. Crit Care Med 23(10): 1638-1652.

Martinod, K., M. Demers, T. A. Fuchs, S. L. Wong, A. Brill, M. Gallant, J. Hu, Y. Wang and D. D. Wagner (2013) Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. Proc Natl Acad Sci U S A 110(21): 8674-8679.

McDonald, B., R. Urrutia, B. G. Yipp, C. N. Jenne and P. Kubes (2012) Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe 12(3): 324-333.

McGhee, J. D. and G. Felsenfeld (1980) Nucleosome structure. Annu Rev Biochem 49: 1115-1156.

McKay, C. J., F. Curran, C. Sharples, J. N. Baxter and C. W. Imrie (1997) Prospective placebo-controlled randomized trial of lexipafant in predicted severe acute pancreatitis. Br J Surg 84(9): 1239-1243.

Merza, M., H. Hartman, M. Rahman, R. Hwaiz, E. Zhang, E. Renstrom, L. Luo, M. Morgelin, S. Regner and H. Thorlacius (2015) Neutrophil Extracellular Traps Induce Trypsin Activation, Inflammation, and Tissue Damage in Mice With Severe Acute Pancreatitis. Gastroenterology 149(7): 1920-1931 e1928.

Merza, M., M. Rahman, S. Zhang, R. Hwaiz, S. Regner, A. Schmidtchen and H. Thorlacius (2014) Human thrombin-derived host defense peptides inhibit neutrophil recruitment and tissue injury in severe acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 307(9): G914-921.

Metzler, K. D., C. Goosmann, A. Lubojemska, A. Zychlinsky and V. Papayannopoulos (2014) A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. Cell Rep 8(3): 883-896.

Michels, A., S. Albanez, J. Mewburn, K. Nesbitt, T. J. Gould, P. C. Liaw, P. D. James, L. L. Swystun and D. Lillicrap (2016) Histones link inflammation and thrombosis through the induction of Weibel-Palade body exocytosis. J Thromb Haemost 14(11): 2274-2286.

Mitroulis, I., K. Kambas, A. Chrysanthopoulou, P. Skendros, E. Apostolidou, I. Kourtzelis, G. I. Drosos, D. T. Boumpas and K. Ritis (2011) Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS One 6(12): e29318.

Mittal, A., A. R. Phillips, M. Middleditch, K. Ruggiero, B. Loveday, B. Delahunt, G. J. Cooper and J. A. Windsor (2009) The proteome of mesenteric lymph during acute pancreatitis and implications for treatment. JOP 10(2): 130-142.

Moggia, E., R. Koti, A. P. Belgaumkar, F. Fazio, S. P. Pereira, B. R. Davidson and K. S. Gurusamy (2017) Pharmacological interventions for acute pancreatitis. Cochrane Database Syst Rev 4: CD011384.

Montecucco, F., F. Mach, S. Lenglet, A. Vonlaufen, A. L. Gomes Quindere, G. Pelli, F. Burger, K. Galan, F. Dallegri, F. Carbone, A. E. Proudfoot, N. Vuilleumier and J. L. Frossard (2014) Treatment with Evasin-3 abrogates neutrophil-mediated inflammation in mouse acute pancreatitis. Eur J Clin Invest 44(10): 940-950.

Mukherjee, R., D. N. Criddle, A. Gukovskaya, S. Pandol, O. H. Petersen and R. Sutton (2008) Mitochondrial injury in pancreatitis. Cell Calcium 44(1): 14-23.

Mukherjee, R., O. A. Mareninova, I. V. Odinokova, W. Huang, J. Murphy, M. Chvanov, M. A. Javed, L. Wen, D. M. Booth, M. C. Cane, M. Awais, B. Gavillet, R. M. Pruss, S. Schaller, J. D. Molkentin, A. V. Tepikin, O. H. Petersen, S. J. Pandol, I. Gukovsky, D. N. Criddle, A. S. Gukovskaya, R. Sutton and N. P. B. R. Unit (2016) Mechanism of mitochondrial permeability transition pore induction and damage in the pancreas: inhibition prevents acute pancreatitis by protecting production of ATP. Gut 65(8): 1333-1346.

Murakami, T., T. Fujita, T. Miyake, A. Ohtsuka, T. Taguchi and A. Kikuta (1993) The insulo-acinar portal and insulo-venous drainage systems in the pancreas of the mouse, dog, monkey and certain other animals: a scanning electron microscopic study of corrosion casts. Arch Histol Cytol 56(2): 127-147.

Murakami, T., S. Hitomi, A. Ohtsuka, T. Taguchi and T. Fujita (1997) Pancreatic insulo-acinar portal systems in humans, rats, and some other mammals: scanning electron microscopy of vascular casts. Microsc Res Tech 37(5-6): 478-488.

Murayama, K. M., B. L. Barent, M. Gruber, A. Brooks, S. Eliason, E. M. Brunt and G. S. Smith (1999) Characterization of a novel model of pancreatic fibrosis and acinar atrophy. J Gastrointest Surg 3(4): 418-425.

Murphy, J. A., D. N. Criddle, M. Sherwood, M. Chvanov, R. Mukherjee, E. McLaughlin, D. Booth, J. V. Gerasimenko, M. G. T. Raraty, P. Ghaneh, J. P. Neoptolemos, O. V. Gerasimenko, A. V. Tepikin, G. M. Green, J. R. Reeve, O.

H. Petersen and R. Sutton (2008) Direct Activation of Cytosolic Ca2+ Signaling and Enzyme Secretion by Cholecystokinin in Human Pancreatic Acinar Cells. Gastroenterology 135(2): 632-641.

Nakamura, Y., T. Kanai, K. Saeki, M. Takabe, J. Irie, J. Miyoshi, Y. Mikami, T. Teratani, T. Suzuki, N. Miyata, T. Hisamatsu, N. Nakamoto, Y. Yamagishi, H. Higuchi, H. Ebinuma, S. Hozawa, H. Saito, H. Itoh and T. Hibi (2013) CCR2 knockout exacerbates cerulein-induced chronic pancreatitis with hyperglycemia via decreased GLP-1 receptor expression and insulin secretion. Am J Physiol Gastrointest Liver Physiol 304(8): G700-707.

Narasaraju, T., E. Yang, R. P. Samy, H. H. Ng, W. P. Poh, A. A. Liew, M. C. Phoon, N. van Rooijen and V. T. Chow (2011) Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. Am J Pathol 179(1): 199-210.

Nathan, J. D., J. Romac, R. Y. Peng, M. Peyton, R. J. Macdonald and R. A. Liddle (2005) Transgenic expression of pancreatic secretory trypsin inhibitor-I ameliorates secretagogue-induced pancreatitis in mice. Gastroenterology 128(3): 717-727.

Neeli, I., S. N. Khan and M. Radic (2008) Histone deimination as a response to inflammatory stimuli in neutrophils. J Immunol 180(3): 1895-1902.

Neeli, I. and M. Radic (2012) Knotting the NETs: analyzing histone modifications in neutrophil extracellular traps. Arthritis Res Ther 14(2): 115.

Neeli, I. and M. Radic (2013) Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. Front Immunol 4: 38.

Nemzek, J. A., G. L. Bolgos, B. A. Williams and D. G. Remick (2001) Differences in normal values for murine white blood cell counts and other hematological parameters based on sampling site. Inflamm Res 50(10): 523-527.

Nieminen, A., M. Maksimow, P. Mentula, L. Kyhala, L. Kylanpaa, P. Puolakkainen, E. Kemppainen, H. Repo and M. Salmi (2014) Circulating cytokines in predicting development of severe acute pancreatitis. Crit Care 18(3): R104.

Niu, S., Z. Bian, A. Tremblay, Y. Luo, K. Kidder, A. Mansour, K. Zen and Y. Liu (2016) Broad Infiltration of Macrophages Leads to a Proinflammatory State in Streptozotocin-Induced Hyperglycemic Mice. J Immunol 197(8): 3293-3301.

Novovic, S., A. M. Andersen, M. Nord, M. Astrand, T. Ottosson, L. N. Jorgensen and M. B. Hansen (2013) Activity of neutrophil elastase reflects the progression of acute pancreatitis. Scand J Clin Lab Invest 73(6): 485-493.

Nugent, K. L. and C. M. Coopersmith (2017) Fluid Management in Sepsis-Is There a Golden Hour (or Two)? Crit Care Med 45(10): 1773-1775.

Nuutinen, P., L. Kivisaari, C. G. Standertskjold-Nordenstam, M. Lempinen and T. Schroder (1986) Microangiography of the pancreas in experimental

oedemic and haemorrhagic pancreatitis. Scand J Gastroenterol Suppl 126: 12-17.

Oiva, J., H. Mustonen, M.-L. Kylänpää, L. Kyhälä, T. Alanärä, S. Aittomäki, S. Siitonen, E. Kemppainen, P. Puolakkainen and H. Repo (2010) Patients with acute pancreatitis complicated by organ failure show highly aberrant monocyte signaling profiles assessed by phospho-specific flow cytometry*. Critical Care Medicine 38(8): 1702-1708.

Oiva, J., H. Mustonen, M. L. Kylanpaa, K. Kuuliala, S. Siitonen, E. Kemppainen, P. Puolakkainen and H. Repo (2013) Patients with acute pancreatitis complicated by organ dysfunction show abnormal peripheral blood polymorphonuclear leukocyte signaling. Pancreatology 13(2): 118-124.

Orabi, A. I., K. A. Muili, D. Wang, S. Jin, G. Perides and S. Z. Husain (2013) Preparation of pancreatic acinar cells for the purpose of calcium imaging, cell injury measurements, and adenoviral infection. J Vis Exp(77): e50391.

Osborn, T. M., M. P. LaMonte and W. R. Gaasch (1999) Intravenous thrombolytic therapy for stroke: a review of recent studies and controversies. Ann Emerg Med 34(2): 244-255.

Otsuki, M., C. Sakamoto, H. Yuu, M. Maeda, S. Morita, A. Ohki, N. Kobayashi, K. Terashi, K. Okano and S. Baba (1979) Discrepancies between the doses of cholecystokinin or caerulein-stimulating exocrine and endocrine responses in perfused isolated rat pancreas. J Clin Invest 63(3): 478-484.

Ou, X., Z. Cheng, T. Liu, Z. Tang, W. Huang, P. Szatmary, S. Zheng, R. Sutton, C. H. Toh, N. Zhang and G. Wang (2015) Circulating Histone Levels Reflect Disease Severity in Animal Models of Acute Pancreatitis. Pancreas 44(7): 1089-1095.

Papayannopoulos, V., K. D. Metzler, A. Hakkim and A. Zychlinsky (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191(3): 677-691.

Park, Y. J. and K. Luger (2008) Histone chaperones in nucleosome eviction and histone exchange. Curr Opin Struct Biol 18(3): 282-289.

Parniczky, A., B. Kui, A. Szentesi, A. Balazs, A. Szucs, D. Mosztbacher, J. Czimmer, P. Sarlos, J. Bajor, S. Godi, A. Vincze, A. Illes, I. Szabo, G. Par, T. Takacs, L. Czako, Z. Szepes, Z. Rakonczay, F. Izbeki, J. Gervain, A. Halasz, J. Novak, S. Crai, I. Hritz, C. Gog, J. Sumegi, P. Golovics, M. Varga, B. Bod, J. Hamvas, M. Varga-Muller, Z. Papp, M. Sahin-Toth, P. Hegyi and G. Hungarian Pancreatic Study (2016) Prospective, Multicentre, Nationwide Clinical Data from 600 Cases of Acute Pancreatitis. PLoS One 11(10): e0165309.

Pastor, C. M., A. Vonlaufen, F. Georgi, A. Hadengue, P. Morel and J. L. Frossard (2006) Neutrophil depletion--but not prevention of Kupffer cell activation--decreases the severity of cerulein-induced acute pancreatitis. World J Gastroenterol 12(8): 1219-1224.

Patel, K., R. N. Trivedi, C. Durgampudi, P. Noel, R. A. Cline, J. P. DeLany, S. Navina and V. P. Singh (2015) Lipolysis of visceral adipocyte triglyceride by pancreatic lipases converts mild acute pancreatitis to severe pancreatitis independent of necrosis and inflammation. Am J Pathol 185(3): 808-819.

Peery, A. F., E. S. Dellon, J. Lund, S. D. Crockett, C. E. McGowan, W. J. Bulsiewicz, L. M. Gangarosa, M. T. Thiny, K. Stizenberg, D. R. Morgan, Y. Ringel, H. P. Kim, M. D. Dibonaventura, C. F. Carroll, J. K. Allen, S. F. Cook, R. S. Sandler, M. D. Kappelman and N. J. Shaheen (2012) Burden of gastrointestinal disease in the United States: 2012 update. Gastroenterology 143(5): 1179-1187 e1171-1173.

Pendharkar, S. A., J. Mathew, J. Zhao, J. A. Windsor, D. J. Exeter and M. S. Petrov (2017) Ethnic and geographic variations in the incidence of pancreatitis and post-pancreatitis diabetes mellitus in New Zealand: a nationwide population-based study. N Z Med J 130(1450): 55-68.

Penttila, A. K., A. Rouhiainen, L. Kylanpaa, H. Mustonen, P. Puolakkainen, H. Rauvala and H. Repo (2016) Circulating nucleosomes as predictive markers of severe acute pancreatitis. J Intensive Care 4: 14.

Pereira, L. F., F. M. Marco, R. Boimorto, A. Caturla, A. Bustos, E. G. De la Concha and J. L. Subiza (1994) Histones interact with anionic phospholipids with high avidity; its relevance for the binding of histone-antihistone immune complexes. Clin Exp Immunol 97(2): 175-180.

Perides, G., G. J. D. van Acker, J. M. Laukkarinen and M. L. Steer (2010) Experimental acute biliary pancreatitis induced by retrograde infusion of bile acids into the mouse pancreatic duct. Nature Protocols 5(2): 335-341.

Perides, G., E. R. Weiss, E. S. Michael, J. M. Laukkarinen, J. S. Duffield and M. L. Steer (2011) TNF-alpha-dependent regulation of acute pancreatitis severity by Ly-6C(hi) monocytes in mice. J Biol Chem 286(15): 13327-13335.

Petrov, M. S., S. Shanbhag, M. Chakraborty, A. R. Phillips and J. A. Windsor (2010) Organ failure and infection of pancreatic necrosis as determinants of mortality in patients with acute pancreatitis. Gastroenterology 139(3): 813-820.

Pfeiler, S., S. Massberg and B. Engelmann (2014) Biological basis and pathological relevance of microvascular thrombosis. Thromb Res 133 Suppl 1: S35-37.

Plusczyk, T., S. Westermann, D. Rathgeb and G. Feifel (1997) Acute pancreatitis in rats: effects of sodium taurocholate, CCK-8, and Sec on pancreatic microcirculation. Am J Physiol 272(2 Pt 1): G310-320.

Ramakrishnan, V. (1997) Histone structure and the organization of the nucleosome. Annu Rev Biophys Biomol Struct 26: 83-112.

Rao, N. V., B. Argyle, X. Xu, P. R. Reynolds, J. M. Walenga, M. Prechel, G. D. Prestwich, R. B. MacArthur, B. B. Walters, J. R. Hoidal and T. P. Kennedy (2010) Low anticoagulant heparin targets multiple sites of inflammation, suppresses

heparin-induced thrombocytopenia, and inhibits interaction of RAGE with its ligands. Am J Physiol Cell Physiol 299(1): C97-110.

Raraty, M., J. Ward, G. Erdemli, C. Vaillant, J. P. Neoptolemos, R. Sutton and O. H. Petersen (2000) Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. Proc Natl Acad Sci U S A 97(24): 13126-13131.

Reaven, E., D. Curry, J. Moore and G. Reaven (1983) Effect of age and environmental factors on insulin release from the perfused pancreas of the rat. J Clin Invest 71(2): 345-350.

Rehermann, B. (2016) Mature peritoneal macrophages take an avascular route into the injured liver and promote tissue repair. Hepatology.

Roberts, S. E., S. Morrison-Rees, A. John, J. G. Williams, T. H. Brown and D. G. Samuel (2017) The incidence and aetiology of acute pancreatitis across Europe. Pancreatology 17(2): 155-165.

Rosin, D. L. and M. D. Okusa (2012) Dying cells and extracellular histones in AKI: beyond a NET effect? J Am Soc Nephrol 23(8): 1275-1277.

Rossaint, J., J. M. Herter, H. Van Aken, M. Napirei, Y. Doring, C. Weber, O. Soehnlein and A. Zarbock (2014) Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. Blood 123(16): 2573-2584.

Rychter, J. W., L. P. van Minnen, A. Verheem, H. M. Timmerman, G. T. Rijkers, M. E. Schipper, H. G. Gooszen, L. M. Akkermans and A. B. Kroese (2009) Pretreatment but not treatment with probiotics abolishes mouse intestinal barrier dysfunction in acute pancreatitis. Surgery 145(2): 157-167.

Ryschich, E., V. Kerkadze, O. Deduchovas, O. Salnikova, A. Parseliunas, A. Marten, W. Hartwig, M. Sperandio and J. Schmidt (2009) Intracapillary leucocyte accumulation as a novel antihaemorrhagic mechanism in acute pancreatitis in mice. Gut 58(11): 1508-1516.

Saffarzadeh, M., C. Juenemann, M. A. Queisser, G. Lochnit, G. Barreto, S. P. Galuska, J. Lohmeyer and K. T. Preissner (2012) Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. PLoS One 7(2): e32366.

Saitoh, T., J. Komano, Y. Saitoh, T. Misawa, M. Takahama, T. Kozaki, T. Uehata, H. Iwasaki, H. Omori, S. Yamaoka, N. Yamamoto and S. Akira (2012) Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe 12(1): 109-116.

Saluja, A., I. Saito, M. Saluja, M. J. Houlihan, R. E. Powers, J. Meldolesi and M. Steer (1985) In vivo rat pancreatic acinar cell function during supramaximal stimulation with caerulein. Am J Physiol 249(6 Pt 1): G702-710.

Saluja, A. K. and M. L. P. Steer (1999) Pathophysiology of pancreatitis. Role of cytokines and other mediators of inflammation. Digestion 60 Suppl 1: 27-33.

Sandoval, D., A. Gukovskaya, P. Reavey, S. Gukovsky, A. Sisk, P. Braquet, S. J. Pandol and S. Poucell-Hatton (1996) The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. Gastroenterology 111(4): 1081-1091.

Savchenko, A. S., J. I. Borissoff, K. Martinod, S. F. De Meyer, M. Gallant, L. Erpenbeck, A. Brill, Y. Wang and D. D. Wagner (2014) VWF-mediated leukocyte recruitment with chromatin decondensation by PAD4 increases myocardial ischemia/reperfusion injury in mice. Blood 123(1): 141-148.

Schauer, C., C. Janko, L. E. Munoz, Y. Zhao, D. Kienhofer, B. Frey, M. Lell, B. Manger, J. Rech, E. Naschberger, R. Holmdahl, V. Krenn, T. Harrer, I. Jeremic, R. Bilyy, G. Schett, M. Hoffmann and M. Herrmann (2014) Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med 20(5): 511-517.

Schietroma, M., B. Pessia, F. Carlei, P. Mariani, F. Sista and G. Amicucci (2016) Intestinal permeability and systemic endotoxemia in patients with acute pancreatitis. Ann Ital Chir 87: 138-144.

Schmidt, J., D. Ebeling, E. Ryschich, J. Werner, M. M. Gebhard and E. Klar (2002) Pancreatic capillary blood flow in an improved model of necrotizing pancreatitis in the rat. J Surg Res 106(2): 335-341.

Schneider, C. A., W. S. Rasband and K. W. Eliceiri (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7): 671-675.

Schneider, L., B. Jabrailova, O. Strobel, T. Hackert and J. Werner (2015) Inflammatory profiling of early experimental necrotizing pancreatitis. Life Sci 126: 76-80.

Semeraro, F., C. T. Ammollo, N. L. Esmon and C. T. Esmon (2014) Histones induce phosphatidylserine exposure and a procoagulant phenotype in human red blood cells. J Thromb Haemost 12(10): 1697-1702.

Semeraro, F., C. T. Ammollo, J. H. Morrissey, G. L. Dale, P. Friese, N. L. Esmon and C. T. Esmon (2011) Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood 118(7): 1952-1961.

Shen, Q., Z. Li, S. Huang, L. Li, H. Gan and X. G. Du (2017) Intestinal mucosal barrier dysfunction in SAP patients with MODS ameliorated by continuous blood purification. Int J Artif Organs: 0.

Shrivastava, P. and M. Bhatia (2010) Essential role of monocytes and macrophages in the progression of acute pancreatitis. World J Gastroenterol 16(32): 3995-4002.

Singh, V. K., T. B. Gardner, G. I. Papachristou, M. Rey-Riveiro, M. Faghih, E. Koutroumpakis, E. Afghani, N. G. Acevedo-Piedra, N. Seth, A. Sinha, N. Quesada-Vazquez, N. Moya-Hoyo, C. Sanchez-Marin, J. Martinez, F. Lluis, D. C. Whitcomb, P. Zapater and E. de-Madaria (2017) An international

multicenter study of early intravenous fluid administration and outcome in acute pancreatitis. United European Gastroenterol J 5(4): 491-498.

Skouras, C., A. J. Hayes, L. Williams, O. J. Garden, R. W. Parks and D. J. Mole (2014) Early organ dysfunction affects long-term survival in acute pancreatitis patients. HPB (Oxford) 16(9): 789-796.

Slaba, I., J. Wang, E. Kolaczkowska, B. McDonald, W. Y. Lee and P. Kubes (2015) Imaging the dynamic platelet-neutrophil response in sterile liver injury and repair in mice. Hepatology 62(5): 1593-1605.

Stefanovic, L., D. A. Brenner and B. Stefanovic (2005) Direct hepatotoxic effect of KC chemokine in the liver without infiltration of neutrophils. Exp Biol Med (Maywood) 230(8): 573-586.

Stigliano, S., F. Belisario, M. Piciucchi, M. Signoretti, G. Delle Fave and G. Capurso (2017) Recurrent biliary acute pancreatitis is frequent in a real-world setting. Dig Liver Dis.

Studley, J. G., R. T. Mathie and L. H. Blumgart (1987) Blood flow measurement in the canine pancreas. J Surg Res 42(1): 101-115.

Sugano, N., M. O. Olson, L. C. Yeoman, B. R. Johnson, C. W. Taylor, W. C. Starbuck and H. Busch (1972) Amino acid sequence of the COOH-terminal portion of the arginine-lysine-rich histone of calf thymus. J Biol Chem 247(11): 3589-3591.

Sun, J. and M. Bhatia (2007) Blockade of neurokinin-1 receptor attenuates CC and CXC chemokine production in experimental acute pancreatitis and associated lung injury. Am J Physiol Gastrointest Liver Physiol 292(1): G143-153.

Szatmary, P. and I. Gukovsky (2016) The Role of Cytokines and Inflammation in the Genesis of Experimental Pancreatitis. Pancreapedia: Exocrine Pancreas Knowledge Base.

Szatmary, P., W. Huang, D. Criddle, A. Tepikin and R. Sutton (2018) Biology, role and therapeutic potential of circulating histones in acute inflammatory disorders. J Cell Mol Med 22(10): 4617-4629.

Szatmary, P., T. Liu, S. T. Abrams, S. Voronina, L. Wen, M. Chvanov, W. Huang, G. Wang, D. N. Criddle, A. V. Tepikin, C. H. Toh and R. Sutton (2017) Systemic histone release disrupts plasmalemma and contributes to necrosis in acute pancreatitis. Pancreatology 17(6): 884-892.

Tadie, J. M., H. B. Bae, S. Jiang, D. W. Park, C. P. Bell, H. Yang, J. F. Pittet, K. Tracey, V. J. Thannickal, E. Abraham and J. W. Zmijewski (2013) HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. Am J Physiol Lung Cell Mol Physiol 304(5): L342-349.

Tagai, C., S. Morita, T. Shiraishi, K. Miyaji and S. Iwamuro (2011) Antimicrobial properties of arginine- and lysine-rich histones and involvement of bacterial outer membrane protease T in their differential mode of actions. Peptides 32(10): 2003-2009.

Talbert, P. B. and S. Henikoff (2010) Histone variants--ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11(4): 264-275.

Tanaka, K., Y. Koike, T. Shimura, M. Okigami, S. Ide, Y. Toiyama, Y. Okugawa, Y. Inoue, T. Araki, K. Uchida, Y. Mohri, A. Mizoguchi and M. Kusunoki (2014) In vivo characterization of neutrophil extracellular traps in various organs of a murine sepsis model. PLoS One 9(11): e111888.

Thomas, M. P., J. Whangbo, G. McCrossan, A. J. Deutsch, K. Martinod, M. Walch and J. Lieberman (2014) Leukocyte protease binding to nucleic acids promotes nuclear localization and cleavage of nucleic acid binding proteins. J Immunol 192(11): 5390-5397.

Tracy, T. F., Jr. (2012) Editorial: Acute pancreatitis and neutrophil gelatinase MMP9: don't get me started! J Leukoc Biol 91(5): 682-684.

Tsuchitani, M., J. Sato and H. Kokoshima (2016) A comparison of the anatomical structure of the pancreas in experimental animals. J Toxicol Pathol 29(3): 147-154.

Tsuji, Y., H. Yamamoto, S. Yazumi, Y. Watanabe, K. Matsueda and T. Chiba (2007) Perfusion computerized tomography can predict pancreatic necrosis in early stages of severe acute pancreatitis. Clin Gastroenterol Hepatol 5(12): 1484-1492.

Tutcu, S., S. Serter, Y. Kaya, E. Kara, N. Nese, G. Pekindil and T. Coskun (2010) Hepatic perfusion changes in an experimental model of acute pancreatitis: evaluation by perfusion CT. Eur J Radiol 75(2): 203-206.

Urban, C. F., U. Reichard, V. Brinkmann and A. Zychlinsky (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. Cell Microbiol 8(4): 668-676.

Van Acker, G. J., E. Weiss, M. L. Steer and G. Perides (2007) Cause-effect relationships between zymogen activation and other early events in secretagogue-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 292(6): G1738-1746.

Van Laethem, J. L., P. Robberecht, A. Resibois and J. Deviere (1996) Transforming growth factor beta promotes development of fibrosis after repeated courses of acute pancreatitis in mice. Gastroenterology 110(2): 576-582.

Varju, I., C. Longstaff, L. Szabo, A. Z. Farkas, V. J. Varga-Szabo, A. Tanka-Salamon, R. Machovich and K. Kolev (2015) DNA, histones and neutrophil extracellular traps exert anti-fibrinolytic effects in a plasma environment. Thromb Haemost 113(6): 1289-1298.

Vasudevan, S., P. Goswami, U. Sonika, B. Thakur, V. Sreenivas and A. Saraya (2018) Comparison of Various Scoring Systems and Biochemical Markers in Predicting the Outcome in Acute Pancreatitis. Pancreas 47(1): 65-71.

von Bruhl, M. L., K. Stark, A. Steinhart, S. Chandraratne, I. Konrad, M. Lorenz, A. Khandoga, A. Tirniceriu, R. Coletti, M. Kollnberger, R. A. Byrne, I. Laitinen, A. Walch, A. Brill, S. Pfeiler, D. Manukyan, S. Braun, P. Lange, J. Riegger, J. Ware, A. Eckart, S. Haidari, M. Rudelius, C. Schulz, K. Echtler, V. Brinkmann, M. Schwaiger, K. T. Preissner, D. D. Wagner, N. Mackman, B. Engelmann and S. Massberg (2012) Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med 209(4): 819-835.

Voronina, S., D. Collier, M. Chvanov, B. Middlehurst, A. J. Beckett, I. A. Prior, D. N. Criddle, M. Begg, K. Mikoshiba, R. Sutton and A. V. Tepikin (2015) The role of Ca2+ influx in endocytic vacuole formation in pancreatic acinar cells. Biochem J 465(3): 405-412.

Wadhwa, V., S. Patwardhan, S. K. Garg, Y. Jobanputra, R. Lopez and M. R. Sanaka (2017) Health Care Utilization and Costs Associated With Acute Pancreatitis. Pancreas 46(3): 410-415.

Wan, M. H., W. Huang, D. Latawiec, K. Jiang, D. M. Booth, V. Elliott, R. Mukherjee and Q. Xia (2012) Review of experimental animal models of biliary acute pancreatitis and recent advances in basic research. HPB (Oxford) 14(2): 73-81.

Wang, H., A. M. Tang, D. Liu, G. Li, L. Ye, X. Li, C. Li and L. Chen (2013) Renoprotective activity of sivelestat in severe acute pancreatitis in rats. Exp Ther Med 6(1): 29-32.

Wang, H. H., A. M. Tang, L. Chen and M. T. Zhou (2012) Potential of sivelestat in protection against severe acute pancreatitis-associated lung injury in rats. Exp Lung Res 38(9-10): 445-452.

Wang, Y., Y. Chen, L. Xin, S. M. Beverley, E. D. Carlsen, V. Popov, K. P. Chang, M. Wang and L. Soong (2011) Differential microbicidal effects of human histone proteins H2A and H2B on Leishmania promastigotes and amastigotes. Infect Immun 79(3): 1124-1133.

Wang, Y., M. Li, S. Stadler, S. Correll, P. Li, D. Wang, R. Hayama, L. Leonelli, H. Han, S. A. Grigoryev, C. D. Allis and S. A. Coonrod (2009) Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 184(2): 205-213.

Wang, Y., W. Liu, X. Liu, M. Sheng, Y. Pei, R. Lei, S. Zhang and R. Tao (2015) Role of liver in modulating the release of inflammatory cytokines involved in lung and multiple organ dysfunction in severe acute pancreatitis. Cell Biochem Biophys 71(2): 765-776.

Ward, J. B., O. H. Petersen, S. A. Jenkins and R. Sutton (1995) Is an elevated concentration of acinar cytosolic free ionised

calcium the trigger for acute pancreatitis? The Lancet 346: 1016-1019.

Warzecha, Z., A. Dembinski, P. Ceranowicz, M. Dembinski, J. Cieszkowski, B. Kusnierz-Cabala, J. W. Naskalski, J. Jaworek, S. J. Konturek, W. W. Pawlik and

R. Tomaszewska (2007) Influence of ischemic preconditioning on blood coagulation, fibrinolytic activity and pancreatic repair in the course of caerulein-induced acute pancreatitis in rats. J Physiol Pharmacol 58(2): 303-319.

Warzecha, Z., A. Dembinski, P. Ceranowicz, M. Dembinski, R. Sendur, J. Cieszkowski, P. Sendur and R. Tomaszewska (2012) Heparin inhibits protective effect of ischemic preconditioning in ischemia/reperfusion-induced acute pancreatitis. J Physiol Pharmacol 63(4): 355-365.

Warzecha, Z., A. Dembinski, P. Ceranowicz, S. J. Konturek, M. Dembinski, W. W. Pawlik, R. Tomaszewska, J. Stachura, B. Kusnierz-Cabala, J. W. Naskalski and P. C. Konturek (2005) Ischemic preconditioning inhibits development of edematous cerulein-induced pancreatitis: involvement of cyclooxygenases and heat shock protein 70. World J Gastroenterol 11(38): 5958-5965.

Watanabe, T., Y. Tsuji, N. Takahashi, T. Yoshida, M. Tamaoki, O. Kikuchi, Y. Watanabe, Y. Kodama, H. Isoda, H. Yamamoto and T. Chiba (2013) Relationship between pancreatic perfusion parameters and clinical complications of severe acute pancreatitis. Pancreas 42(1): 180-182.

Wen, L., S. Voronina, M. A. Javed, M. Awais, P. Szatmary, D. Latawiec, M. Chvanov, D. Collier, W. Huang, J. Barrett, M. Begg, K. Stauderman, J. Roos, S. Grigoryev, S. Ramos, E. Rogers, J. Whitten, G. Velicelebi, M. Dunn, A. V. Tepikin, D. N. Criddle and R. Sutton (2015) Inhibitors of ORAI1 Prevent Cytosolic Calcium-Associated Injury of Human Pancreatic Acinar Cells and Acute Pancreatitis in 3 Mouse Models. Gastroenterology 149(2): 481-492 e487.

Werge, M., S. Novovic, P. N. Schmidt and L. L. Gluud (2016) Infection increases mortality in necrotizing pancreatitis: A systematic review and meta-analysis. Pancreatology 16(5): 698-707.

Wildhagen, K. C., M. A. Wiewel, M. J. Schultz, J. Horn, R. Schrijver, C. P. Reutelingsperger, T. van der Poll and G. A. Nicolaes (2015) Extracellular histone H3 levels are inversely correlated with antithrombin levels and platelet counts and are associated with mortality in sepsis patients. Thromb Res 136(3): 542-547.

Williams, F. M., P. D. Collins, M. Tanniere-Zeller and T. J. Williams (1990) The relationship between neutrophils and increased microvascular permeability in a model of myocardial ischaemia and reperfusion in the rabbit. Br J Pharmacol 100(4): 729-734.

Williams, J. A., M. Korc and R. L. Dormer (1978) Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. Am J Physiol 235(5): 517-524.

Wu, B. U., M. Batech, M. Quezada, D. Lew, K. Fujikawa, J. Kung, L. H. Jamil, W. Chen, E. Afghani, S. Reicher, J. Buxbaum and S. J. Pandol (2017) Dynamic Measurement of Disease Activity in Acute Pancreatitis: The Pancreatitis Activity Scoring System. Am J Gastroenterol 112(7): 1144-1152.

Wyncoll, D. L. and R. J. Beale (1998) Prospective placebo-controlled randomized trial of lexipafant in predicted severe acute pancreatitis. Br J Surg 85(2): 279-280.

Xu, J., Y. Cui and X. Tian (2017) Early Continuous Veno-Venous Hemofiltration Is Effective in Decreasing Intra-Abdominal Pressure and Serum Interleukin-8 Level in Severe Acute Pancreatitis Patients with Abdominal Compartment Syndrome. Blood Purif 44(4): 276-282.

Xu, J., X. Zhang, R. Pelayo, M. Monestier, C. T. Ammollo, F. Semeraro, F. B. Taylor, N. L. Esmon, F. Lupu and C. T. Esmon (2009) Extracellular histones are major mediators of death in sepsis. Nat Med 15(11): 1318-1321.

Xu, P., X. J. Zhou, L. Q. Chen, J. Chen, Y. Xie, L. H. Lv and X. H. Hou (2007) Pioglitazone attenuates the severity of sodium taurocholate-induced severe acute pancreatitis. World J Gastroenterol 13(13): 1983-1988.

Xu, Z., Y. Huang, P. Mao, J. Zhang and Y. Li (2015) Sepsis and ARDS: The Dark Side of Histones. Mediators Inflamm 2015: 205054.

Yadav, A. K., R. Sharma, D. Kandasamy, A. S. Bhalla, S. Gamanagatti, D. N. Srivastava, A. D. Upadhyay and P. K. Garg (2015) Perfusion CT: can it predict the development of pancreatic necrosis in early stage of severe acute pancreatitis? Abdom Imaging 40(3): 488-499.

Yadav, D. and A. B. Lowenfels (2013) The epidemiology of pancreatitis and pancreatic cancer. Gastroenterology 144(6): 1252-1261.

Yeoman, L. C., M. O. Olson, N. Sugano, J. J. Jordan, D. W. Taylor, W. C. Starbuck and H. Busch (1972) Amino acid sequence of the center of the arginine-lysine-rich histone from calf thymus. The total sequence. J Biol Chem 247(19): 6018-6023.

Yokoe, M., T. Takada, T. Mayumi, M. Yoshida, S. Isaji, K. Wada, T. Itoi, N. Sata, T. Gabata, H. Igarashi, K. Kataoka, M. Hirota, M. Kadoya, N. Kitamura, Y. Kimura, S. Kiriyama, K. Shirai, T. Hattori, K. Takeda, Y. Takeyama, M. Hirota, M. Sekimoto, S. Shikata, S. Arata and K. Hirata (2015) Japanese guidelines for the management of acute pancreatitis: Japanese Guidelines 2015. J Hepatobiliary Pancreat Sci 22(6): 405-432.

Yoon, S. B., M. H. Choi, I. S. Lee, C. H. Lim, J. S. Kim, Y. K. Cho, J. M. Park, B. I. Lee, Y. S. Cho and M. G. Choi (2017) Impact of body fat and muscle distribution on severity of acute pancreatitis. Pancreatology 17(2): 188-193.

Young, E. (2008) The anti-inflammatory effects of heparin and related compounds. Thromb Res 122(6): 743-752.

Young, R. L., K. C. Malcolm, J. E. Kret, S. M. Caceres, K. R. Poch, D. P. Nichols, J. L. Taylor-Cousar, M. T. Saavedra, S. H. Randell, M. L. Vasil, J. L. Burns, S. M. Moskowitz and J. A. Nick (2011) Neutrophil extracellular trap (NET)-mediated killing of Pseudomonas aeruginosa: evidence of acquired resistance within the CF airway, independent of CFTR. PLoS One 6(9): e23637.

Yu, Y., S. G. Wei, R. M. Weiss and R. B. Felder (2017) TNF-alpha receptor 1 knockdown in the subfornical organ ameliorates sympathetic excitation and cardiac hemodynamics in heart failure rats. Am J Physiol Heart Circ Physiol 313(4): H744-H756.

Zampronio, A. R., M. C. Melo, C. A. Silva, I. R. Pela, S. J. Hopkins and G. E. Souza (1994) A pre-formed Pyrogenic Factor Released by Lipopolysaccharide Stimulated Macrophages. Mediators Inflamm 3(5): 365-373.

Zaninovic, V., A. S. Gukovskaya, I. Gukovsky, M. Mouria and S. J. Pandol (2000) Cerulein upregulates ICAM-1 in pancreatic acinar cells, which mediates neutrophil adhesion to these cells. Am J Physiol Gastrointest Liver Physiol 279(4): G666-676.

Zhang, J. X., S. C. Dang, Y. Zhang, X. Sha, L. R. Zhang, C. S. Wei, M. Chen and D. L. Jiang (2010) MRI shows clodronate-liposomes attenuating liver injury in rats with severe acute pancreatitis. Hepatobiliary Pancreat Dis Int 9(2): 192-200.

Zhang, L., K. Mizumoto, N. Sato, T. Ogawa, M. Kusumoto, H. Niiyama and M. Tanaka (1999) Quantitative determination of apoptotic death in cultured human pancreatic cancer cells by propidium iodide and digitonin. Cancer Lett 142(2): 129-137.

Zhang, Y., L. Guan, J. Yu, Z. Zhao, L. Mao, S. Li and J. Zhao (2016) Pulmonary endothelial activation caused by extracellular histones contributes to neutrophil activation in acute respiratory distress syndrome. Respir Res 17(1): 155.

Zhang, Y., W. Wu, L. Dong, C. Yang, P. Fan and H. Wu (2016) Neutrophil to lymphocyte ratio predicts persistent organ failure and in-hospital mortality in an Asian Chinese population of acute pancreatitis. Medicine (Baltimore) 95(37): e4746.

Zhou, Z. G., Y. D. Chen, W. Sun and Z. Chen (2002) Pancreatic microcirculatory impairment in experimental acute pancreatitis in rats. World J Gastroenterol 8(5): 933-936.

10 Appendix – Publications arising from this thesis

Reviews

Szatmary P, Huang, W, Criddle D, Tepikin A, Sutton R; 'Biology, Role and Therapeutic Potential of Circulating Histones in Acute Inflammatory Disorders.' Cell Molec Med 2018; ePub ahead of print.

Szatmary P and Gukovsky I; 'The Role of Cytokines and Inflammation in the Genesis of Experimental Pancreatitis. Pancreapedia: Exocrine Pancreas Knowledge Base 2016 and book chapter in 'Acute Pancreatitis' edited by John Williams, Dec 2016.

Research articles

Szatmary P, Liu T, Abrams ST, Voronina S, Wen L, Chvanov M, Huang W, Wang G, Criddle DN, Tepikin AV, Toh CH, Sutton R; 'Systemic histones release disrupts plasmalemma and contributes to necrosis in acute pancreatitis.' Pancreatology 2017; 17(6):884-92.

Liu T, Huang W, **Szatmary P**, Abrams ST, Alhamdi Y, Lin Z, Greenhalf W, Wang G, Sutton R, Toh CH; 'Accuracy of circulating histones in predicting persistent organ failure and mortality in patients with acute pancreatitis.' Br J Surg 2017; 104(9):1215-1225.

Conference poster and oral presentations

Szatmary P, Liu T, Criddle D, Tepikin A, Sutton R: 'Effect of Heparins of Histone Catabolism and Cellular Injury in Acute Pancreatitis.' American Pancreatic Association Annual Meeting 2016, Boston, MA, USA

Szatmary P, Taylor A, Poptani H, Criddle D, Tepikin A, Sutton R: 'Novel Methods for the Measurement of Blood Flow in Experimental Acute Pancreatitis.' American Pancreatic Association Annual Meeting 2016, Boston, MA, USA

Szatmary P, Liu T, Abrams S, Wen L, Wang G, Toh CH, Criddle DN, Tepikin A, Sutton R: 'Circulating Histones Target Acinar Cells and Exacerbate Injury in Experimental Acute Pancreatitis.' American Pancreatic Association Annual Meeting 2015, San Diego, CA, USA

Szatmary P, Huang W, Liu T, de la Iglesia-Garcia D, Parente S, Primo G, Greenhalf W, Sutton R: 'Pattern and Impact of Organ Failure on Human Acute

Pancreatitis.' American Pancreatic Association Annual Meeting 2015, San Diego, CA, USA

Szatmary P, Huang W, Liu T, de la Iglesia-Garcia D, Parente S, Primo G, Greenhalf W, Sutton R: 'Pattern and Impact of Infective Complications on Human Acute Pancreatitis.' American Pancreatic Association Annual Meeting 2015, San Diego, CA, USA

Szatmary P: 'Pattern and Impact of Organ Failure and Infective Complications in Acute Pancreatitis.' International Association of Pancreatologists Annual Meeting 2015, Shanghai, China

Szatmary P, Liu T, Wen L, Huang W, Awais M, Wang G, Ghaneh P, Halloran C, Neoptolemos JP, Raraty M, Toh CH, Sutton R: 'The Role of Neutrophil Extracellular Traps in Acute Pancreatitis'. American Pancreatic Association Annual Meeting 2014, Hawaii, USA

Szatmary P, Liu T, Huang W, Wang G, Latawiec D, Davies K, Ghaneh P, Halloran C, Neoptolemos JP, Raraty M, Toh CH, Sutton R: 'Use of Citrullinated Histones and Circulating Nucleosomes in Early Prediction of Organ Failure in Acute Pancreatitis'. American Pancreatic Association Annual Meeting 2014, Hawaii, USA